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Haplotype diversity in the interleukin-4 gene is not associated with HIV-1 transmission and AIDS progression

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Abstract Interleukin-4 (IL-4) is a pleiotropic cytokine produced primarily by activated CD4⁺ T lymphocytes, mast cells, and basophils. It modulates the functions of a variety of cell types involved with the immune response.

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This cytokine differentially regulates two major HIV-1 coreceptors and activates viral expression, and is thus a reasonable candidate gene for analyses in HIV-1/AIDS cohort studies. Population genetic variation in five single nucleotide polymorphisms (SNPs) in the 5' region of the *IL-4* gene was assessed in five racial groups. Neutrality tests reveal that the populations are evolving in accord with the infinite-sites model. However, coalescent simulations suggest greater haplotype diversity among African Americans than expected. This increased variation is presumably attributable to recombination or gene conversion. Genetic epidemiological analyses were conducted among European American and African American participants enrolled in five USA-based HIV-1/AIDS cohorts. One SNP, -589T, known to influence *IL-4* transcription was previously shown to be associated with HIV-1/AIDS in both Japanese and French populations. Present analyses failed to identify any significant associations with HIV-1 infection or progression to AIDS.

Keywords HIV-1 · AIDS · Interleukin-4 · Haplotype diversity · Recombination hotspot

Introduction

Interleukin-4 (IL-4) is a multifunctional cytokine critically involved in regulating various aspects of the immune system. It regulates the growth and differentiation of hematopoietic cells, the production of IgE (Del Prete et al. 1988) the modulation of IgG isotype switching (Vitetta et al. 1985), and the adhesive properties of endothelial cells (Schleimer et al. 1992). IL-4 has also been implicated in HIV-1 pathogenesis. It plays a pivotal role in the development of naive T cells by inducing the TH2 lymphocyte state (Paul 1991). An effective, early immune response is characterized by anti-HIV-1 CTLs and a TH1 cytokine profile [production of gamma interferon (IFN- γ), IL-2, and IL-12]. In contrast, the TH2 state, determined predominantly by IL-4 and IL-10 is associated with a poor CTL response, ineffective

immune regulation of HIV-1 replication, and more rapid progression to CD4⁺ T-cell depletion and AIDS-defining conditions (Galli et al. 1998; Klein et al. 1997; Valentin et al. 1998; Wasik et al. 1997). IL-4 may also modify viral replication. Treatment of CD4⁺ T cells with IL-4 down-regulates CCR5, the primary coreceptor for R5 strains of HIV-1, and up-regulates CXCR4, the coreceptor utilized by the more pathogenic X4 strains (Valentin et al. 1998). Up-regulation of CXCR4 has been correlated with enhanced viral replication and higher levels of infected CD4⁺ T cells (Wang et al. 1998). IL-4 also supports the replication of syncytia inducing (SI) laboratory strains in primary T cells. SI variants usually emerge late in infection and are associated with decreased CD4⁺ levels and more rapid disease progression (Connor et al. 1993).

A C→T single nucleotide polymorphism (SNP) at -589 in the *IL-4* promoter was associated with increased transcription and elevated serum IgE levels in asthmatic families (Rosenwasser et al. 1995). It was subsequently reported that the -589T/T genotype was associated with increased rates of SI strain acquisition and elevated serum IgE levels in HIV-1 infected Japanese patients (Nakayama et al. 2000). Recently, the -589T allele was found to be associated with delayed progression to AIDS and death, and decreased viral load in the French SEROCO cohort (Nakayama et al. 2002). Given the importance of IL-4 in mediating the immune system and the two reported influences of -589T on HIV-1/AIDS pathogenesis, we performed an extensive population genetic and epidemiological investigation of five SNPs, including -589C/T, located in the 5' end of the *IL-4* gene.

Materials and methods

SNP identification

SNPs were discovered using the single-strand conformational polymorphism technique (SSCP) and direct sequencing. Six pairs of oligonucleotides were synthesized using the GenBank *IL-4* genomic sequence M23442. A total of 462 bp of coding (all four exons) and 1,860 bp of non-coding (about 1,200 bp upstream of the initiation codon and 75 bp flanking each exon) DNA were screened from 72 clinically unaffected European Americans and 72 African Americans, as described (Modi et al. 2001). PCR products from each primer pair were sequenced in a subset of individuals.

Genotyping

The PCR-RFLP method was used to genotype two SNPs and Taq-Man allelic discrimination (Applied Biosystems) for three. Primers 5'-CAATGTAACTCATTTTCCC-3' and 5'-GAAATACTGAGCATCACC-3' generate a 325-bp product that contains two SNPs. A C→T change (AATTG(C/T)CTCAC) at position -33 (where the initiation codon ATG = +1) produces fragments of 325 bp (common allele in European Americans) or 224 and 101 bp (variant allele) when digested with *Bsm*AI. An A→G substitution (CTGCT(A/G)GCATG) at base-pair 45 produces bands of 178 and 147 bp (common allele) and 325 bp (variant allele) after digestion with *Bfa*I. RFLP genotyping was conducted as described (Modi et al. 2001).

One pair of Taq-Man PCR primers, 5'-CAATGAGCACCT-TATTGTGTCCA-3' and 5'-GGAACCTAAACACATCCTCAGC-

TAA-3', was used to genotype the -1136G/A and -1098T/G SNPs. The allelic discrimination probes for -1136G/A were 5'-6FAM-TCCTAGTCAGTGCCCCACCACCC-TAMARA-3' and 5'-VIC-TCTTAGTCAGTGTTCCCACCACCCG-TAMARA-3'. The TURBO (propylene T) allelic discrimination probes for -1098T/G were 5'-6FAM-AAGTTGGTAAGACTGTAGCTCTTTTTTC-TAMARA-3' and 5'-VIC-AGTTGGTAAAGACGGTAGCTCTTTTT-TAMARA-3'. The PCR primers for -589C/T were 5'-CCTGATACGAC-CTGTCCTTCTCAA-3' and 5'-ATTTGTTGTAATGCAGTCCT-CCTG-3'. The allelic discrimination probes were 5'-6FAM-CTT-GGGAGAACATTGTCCCCAGTG-TAMARA-3' and 5'-VIC-TTGGGAGAACATTGTTCCCCAGTGC-TAMARA-3'. PCR amplification used ABI Taq-Man buffer, and cycling conditions included an initial denaturation at 95 °C for 10 min, followed by 40 cycles of 95 °C for 15 s and 62 °C for 1 min. Reactions were read in an ABI 7700 plate reader.

Study population

The study group includes 951 seroconverters, 1642 seroprevalents, and 641 seronegatives for a total of 3,334 [European Americans (EA)=1,904; African Americans (AA)=1,297; Hispanic (HS)=133] from five USA-based natural history AIDS cohorts: AIDS Link to the Intravenous Experience [ALIVE (Vlahov et al. 1998)], Hemophilia Growth and Development Study [HGDS (Hilgartner et al. 1993)], Multicenter Hemophiliac Cohort [MHCS (Goedert et al. 1989)], Multicenter AIDS Cohort Study [MACS (Detels et al. 1994; Kaslow et al. 1987)] and San Francisco City Clinic Study [SFCC (Buchbinder et al. 1994)]. In addition, genotypes were obtained from 140 unrelated, healthy adult Zapotec, Mixe, and Mixtec Meso-American Amerindians residing in rural villages in the state of Oaxaca, Mexico and from 143 Han Chinese from Beijing, Peoples Republic of China. Informed consent was obtained from all study participants. Seroconversion date was estimated as the midpoint between the last seronegative and first seropositive HIV-1 antibody test dates (mean interval 0.79 years, range 0.07–3.0 years). High-risk exposed HIV-1 uninfected (HREU) subjects ($n=271$) were those with a high-risk of exposure through sharing of injection equipment (Vlahov and Polk 1988), anal receptive sex with multiple partners (Detels et al. 1994; Rutherford et al. 1990), or transfusions with factor VIII clotting factor before 1984 when heat treatment was initiated (Salkowitz et al. 2001). All participants were censored as of December 1996, prior to widespread use of highly active anti-retroviral therapy (HAART).

Statistical analyses

The computation of Tajima's *D* (Tajima 1989), a statistic that compares independent estimates of the neutral parameter (π and θ), was carried out using DnaSP 3.51 (Rozas and Rozas 2000). The four-gamete test measured recombination between pairs of non-overlapping SNPs (Hudson and Kaplan 1985). This test assumes that only three of four possible haplotypes exist in the absence of recombination, recurrent mutation, or gene conversion. RM represents the minimum number of observed recombination events in a population. Evolutionary trees depicting relationships among haplotypes were constructed under the parsimony criterion using PAUP* (Swofford 1998).

Haplotypes were estimated in compound heterozygotes using the expectation maximization (E-M) algorithm (Long et al. 1995). A coalescent simulation with 10,000 replications was used to derive expected values and confidence intervals for the number of haplotypes and haplotype diversity conditioned on sample size and number of segregating sites under an infinite-sites model without recombination (Hudson 1990). These calculations were implemented using DnaSP 3.51.

Defined categorical analyses compared genotype and haplotype frequencies between HIV-1 seroconverters and HREU for associations with HIV-1 infection using a two-tailed Fisher's exact test.

Kaplan-Meier non-parametric survival analyses and Cox proportional hazards regression assessed rates of disease progression among HIV-1-infected seroconvertors. Analyses were conducted using SAS software (SAS Institute, Cary, N.C.). Three endpoints reflecting advancing HIV-1 disease progression were evaluated: time to less than 200 CD4⁺ cells per mm³ (CD4<200); AIDS 1987, as defined by the Centers for Disease Control and Prevention (Centers for Disease Control 1987); AIDS-related death during follow-up for an HIV-1-infected participant. Cox analyses were performed both unadjusted (using only *IL-4* genetic factors) or adjusted (including, in addition to *IL-4* genotypes or haplotypes, additional AIDS-modifying genetic factors as covariates: for EA, *CCR5-Δ32*, *CCR2-64I*, *SDF1-3'A*, *HLA-B*27*, *HLA-B*57*, *HLA-B*35Px*, and *HLA* class I zygosity; for AA, *HLA-B*57*, *HLA-B*35Px*, and *HLA* class I zygosity (Carrington et al. 1999; Dean et al. 1996; Gao et al. 2001; Smith et al. 1997; Winkler et al. 1998). Participants were stratified by ethnic group, sex (7.6% female), and age at seroconversion: 0<20, 20–40, and over 40 years.

Results

SNP and haplotype diversity

SSCP analyses identified seven polymorphic sites, two of which were singletons in African Americans. The remaining five were found at appreciable frequencies: two are novel (–1136A and 45G), while three were reported previously [–1098G (Nakayama et al. 2000), –589T (Borish et al. 1994), –33T (Takabayashi et al. 1999)]. The sequence contexts and positions relative to the translation start site (given as +1) of the five primary variants are: a G→A change at position –1136 (GTGGG(G/A)CACTG); a T→G at position –1098 (AAGAC(T/G)GTAGC); a C→T at position –589 (ATTGT(C/T)CCCCA); a C→T at position –33

(AATTG(C/T)CTCAC); and an A→G at position 45 (CTGCT(A/G)GCATG). This latter SNP occurs at the third position of codon 15 (CTA→CTG) in exon 1 and does not change the amino acid leucine.

Frequencies for the five SNPs are given in Fig 1. Variants –1136A and 45G were common only in African Americans, while the other three were polymorphic in all racial groups, including Japanese (Nakayama et al. 2000). The nucleotide diversity estimate, π , for European Americans (0.00027) was lower, while that for African Americans (0.0008) (Table 1) was comparable to values reported for other genes (Stephens et al. 2001). Tajima's D was positive in both races; however, neither value was significant (Table 1).

Pair-wise comparisons between non-overlapping SNPs revealed the presence of all four phase-known haplotypes in each of three intervals: in the 556 bp separating –1098G and –589T in African Americans, in the 509 bp separating –589T and –33T in African Americans, European Americans, and Hispanics, and in the 78 bp separating –33C and 45G in African Americans. In contrast, only two haplotypes were observed between –589T and –33T in Chinese and Meso-American Amerindians (Fig. 1; Table 2).

The E-M algorithm estimated haplotypes containing all five SNPs. Three to five haplotypes were observed in European Americans, Hispanics, Chinese, and Meso-American Amerindians, while 12 were noted in African Americans (Fig. 1). The coalescent simulation generated 99% confidence intervals for expected haplotype numbers, K , and diversity, H_d (Table 1). Among African Americans, a statistically significant ($P<0.01$) increased number of

Fig. 1 Molecular map of the human *IL-4* gene showing locations of five SNPs. Allele and haplotype frequencies are given for European Americans (EA), African Americans (AA), and Hispanic (HS), Chinese (CH) and Meso-American Amerindians (ME). Japanese (JA) data are from (Nakayama et al. 2000). Arrows indicate intervals where all four haplotypes were observed in the four-gamete test (ND not determined)

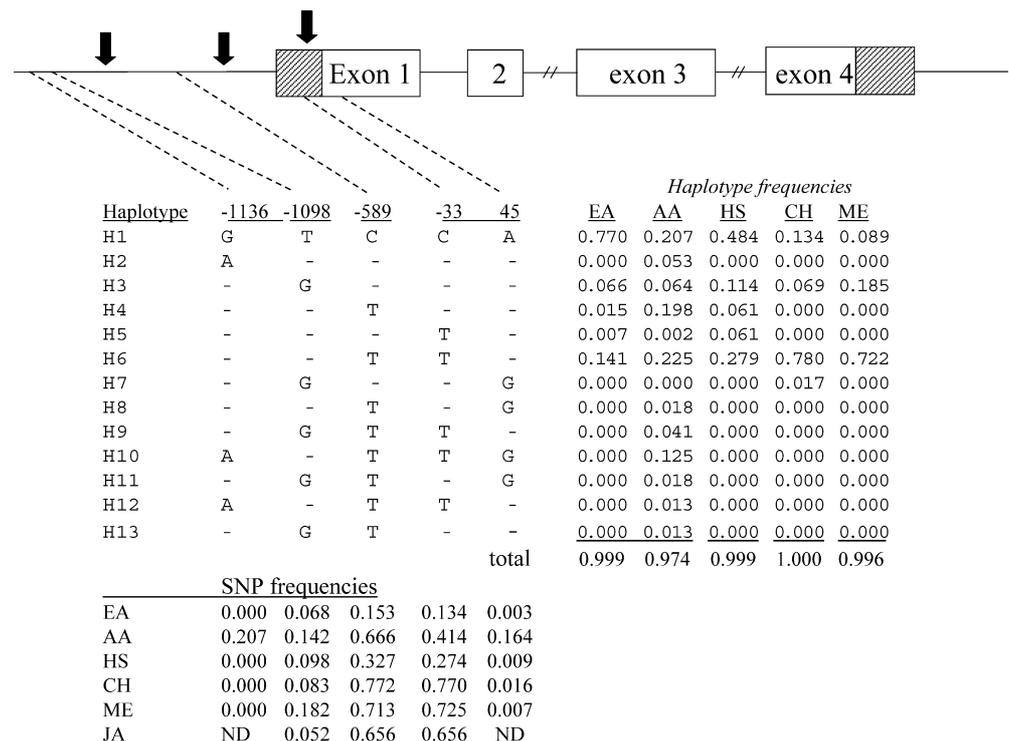
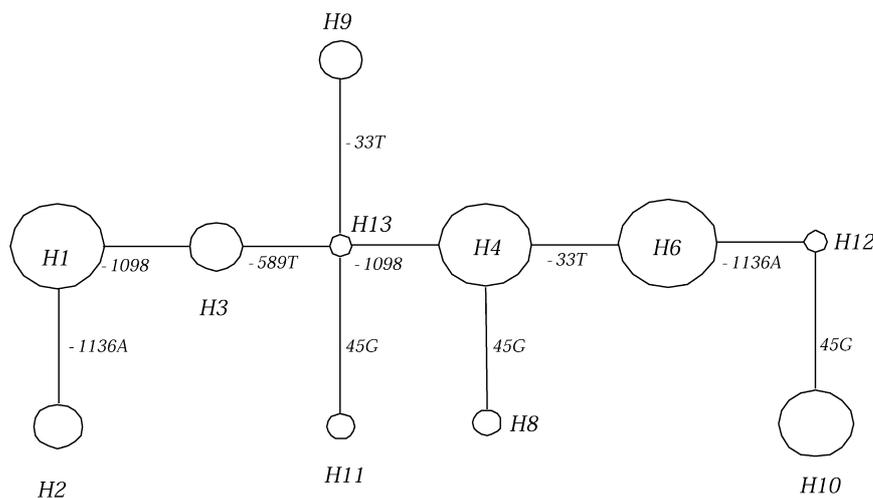


Table 1 Summary statistics of population variation in the *IL-4* gene

Parameter	Symbol	European Americans ^a	African Americans ^a	Hispanics	Chinese	Mesoindians
Number of segregating sites	<i>S</i>	3	7			
Watterson's <i>theta</i> (1975)	θ	0.00027	0.00062			
nucleotide diversity	π	0.00028	0.00080			
Tajima's <i>D</i> (1989)	<i>D</i>	0.127	0.709			
Observed no. of haplotypes (99% CI)	<i>K</i>	5 (3–6)	12* (3–6)	5 (3–6)	4 (3–6)	3 (3–6)
Observed haplotype diversity (99% CI)	<i>Hd</i>	0.383 (0.055–0.780)	0.842 (0.063–0.771*)	0.668 (0.055–0.775)	0.369 (0.063–0.7850)	0.437 (0.063–0.779)

* $P < 0.01$ ^a A total of 2,322 bp were surveyed in 144 European American and 144 African American chromosomes**Table 2** Phase-known haplotype counts among adjacent SNP pairs in the *IL-4* gene. The presence of all four haplotypes indicates that intragenic recombination, gene conversion and/or recurrent mutation has taken place

<i>-1098G/-589T</i>		<i>-589T/-33T</i>		<i>-589T/-33T</i>		<i>-589T/-33T</i>	
African Americans		European Americans		African Americans		Hispanics	
(642 total haplotypes)		(1,794 total haplotypes)		(676 total haplotypes)		(106 total haplotypes)	
TC	154	CC	1315	CC	126	CC	46
TT	349	CT	13	CT	6	CT	5
GC	10	TC	29	TC	175	TC	7
GT	33	TT	39	TT	181	TT	
<i>-589T/-33T</i>		<i>-589T/-33T</i>		<i>-33T/45</i>			
Chinese		Mesoindians		African Americans			
(242 total haplotypes)		(280 total haplotypes)		(1,154 total haplotypes)			
CC	16	CC	24	CA	546		
CT	0	CT	0	CG	31		
TC	0	TC	0	TA	330		
TT	150	TT	150	TG	67		

Fig. 2 A representative parsimony tree for African American *IL-4* haplotypes (except *H7*) following an analysis of 632 chromosomes. Haplotypes correspond to Table 1. The area of each circle is proportional to haplotype frequency. SNP changes occur at the branches indicated

haplotypes, 12, and diversity, 0.842, were observed. The haplotype diversity among African American is represented as an un-rooted tree in Fig. 2. Convergent changes at each SNP resulted in over 100 equally parsimonious solutions.

HIV-1/AIDS association analyses

To determine if any of the five *IL-4* variant alleles, genotypes or haplotypes were associated with HIV-1 transmission, we compared the HREU group with HIV-1-infected seroconverters. No significant distortions in allele, genotype, or haplotype frequencies were noted between the HREU and HIV-1-infected groups for

Table 3 Dominant *IL-4* genotype frequencies and results from comparing HIV-1 infected seroconvertors (*SC*) with high-risk seronegatives (*HREU*). Odds ratios (*OR*), 95% confidence intervals (*CI*), and *P* values are from two-tailed Fisher's exact test

European Americans					
SNP	SC (n=611)	HREU (n=152)	OR	CI	<i>P</i>
-1098G	0.123	0.140	0.86	0.49–1.57	0.57
-589T	0.309	0.270	1.21	0.79–1.86	0.42
-33T	0.285	0.253	1.18	0.76–1.84	0.47
African Americans					
SNP	SC (n=254)	HREU (n=78)	OR	CI	<i>P</i>
-1136A	0.359	0.380	0.91	0.51–1.66	0.78
-1098G	0.286	0.208	1.52	0.78–3.12	0.22
-589T	0.894	0.863	1.33	0.54–3.10	0.53
-33T	0.689	0.679	1.04	0.58–1.85	0.89
45G	0.335	0.295	1.20	0.67–2.19	0.58

Table 4 Cox proportional hazards regression analysis for progression to three AIDS outcomes for dominant *IL-4* genotypes among HIV-1-positive seroconvertors (*SC*). Relative hazards (*RH*), 95% confidence intervals (*CI*) and *P* values are given

European Americans (n=611)									
SNP	CD4<200			AIDS-87			Death		
	RH	CI	<i>P</i>	RH	CI	<i>P</i>	RH	CI	<i>P</i>
-1098G	1.16	0.84–1.61	0.36	1.12	0.78–1.62	0.53	1.16	0.79–1.69	0.45
-589T	0.64	0.30–1.37	0.25	1.24	0.96–1.61	0.10	1.25	0.95–1.65	0.12
-33T	1.11	0.86–1.44	0.41	.30	0.99–1.70	0.06	1.39	1.04–1.85	0.02*
African Americans (n=254)									
SNP	CD4<200			AIDS-87			Death		
	RH	CI	<i>P</i>	RH	CI	<i>P</i>	RH	CI	<i>P</i>
-1136A	0.97	0.62–1.52	0.90	1.24	0.72–2.13	0.45	1.15	0.55–2.39	0.70
-1098G	1.42	0.89–2.27	0.14	1.32	0.74–2.33	0.35	1.22	0.57–2.61	0.60
-589T	0.91	0.47–1.74	0.77	1.13	0.50–2.54	0.77	0.67	0.35–1.64	0.38
-33T	0.78	0.50–1.20	0.26	1.11	0.63–1.94	0.73	0.61	0.30–1.21	0.16
45G	1.00	0.65–1.54	0.99	0.90	0.52–1.56	0.72	0.65	0.30–1.42	0.28

African Americans or European Americans. SNP *-1098G* in African Americans showed the greatest genotype frequency difference between HIV-1-infected seroconvertors (29%) and HREU (21%) participants ($P<0.22$) (Table 3).

Cox proportional hazards regression assessed the association between genotype and rates of disease progression to HIV-1 outcomes (Table 4). In European Americans, *-33T* was significantly associated with an increased rate of progression to death ($RH=1.39$, $P=0.02$); however, no other significant associations were observed in either racial group.

Discussion

Polymorphism levels and haplotype diversity

This study assessed population variation and haplotype diversity in the *IL-4* gene. This information was then used to examine the potential influence of *IL-4* genetic variants on HIV-1/AIDS pathogenesis. Results indicate low to average levels of nucleotide diversity and positive values of Tajima's *D*. Positive *D* values indicate excess heterozygosity relative to the number of segregating sites (Przeworski et al. 2000; Tajima 1989), or a deficiency in the number of low-frequency sites due to balancing selection or population subdivision.

Since all of the tests were non-significant, one can conclude that the populations are evolving as predicted by the infinite-sites model and that selection is not acting on this region.

However, the coalescent simulations revealed greater haplotype diversity in African Americans than was expected under the infinite-sites model. This conclusion was echoed in the results of the four-gamete test, where all four gametes were observed in each of three short intervals, namely 556 bp, 509 bp, and 78 bp. Three different molecular mechanisms: recombination (a single, reciprocal crossing-over event), gene conversion (non-reciprocal transfer of DNA from one chromosome to another) or recurrent mutation (repeated mutation at a site) could explain the results. It is not possible to favor recombination or conversion over the other; however recurrent mutation can seemingly be ruled out, since only $-1098T/G$, having a CpG dinucleotide, would be classified as a mutagenic position (Todorova and Danieli 1997).

If recombination is responsible for the observed excess of African American haplotypes in the *IL-4* gene, then a minimum of three recombination events ($RM=3$) in the 1,143-bp region are necessary. This represents an average of $RM=1$ every 381 bp, a level of recombination much higher than that found in two recent genome-wide surveys where $RM=55$ in 71,824 bp from 15 different regions or $RM=1$ every 13,056 bp (Ardlie et al. 2001), and where $RM=7$ in 22,454 bp or $RM=1$ every 3,208 bp (Przeworski et al. 2000). However, a recombination hotspot in the lipoprotein lipase gene where $RM=29$ in 9,700 bp, or $RM=1$ every 334 bp, was reported (Templeton et al. 2000). Alternatively, one might argue in favor of gene conversion for increasing haplotype diversity. One recent study (Ardlie et al. 2001) measured LD from resequencing surveys; and using the four-gamete test concluded that gene conversion was responsible for the observed incomplete LD among a significant number of closely spaced SNPs. However, one must also realize that admixture among African Americans, and population subdivision and prolonged history among Africans have contributed more broadly to increased genetic diversity in these racial groups.

The presence of only two haplotypes among Chinese and Meso-American Amerindians in the $-589T$ and $-33T$ interval reflects a reduction in haplotype diversity that may be attributable to selection or population history. One reasonable explanation would be the occurrence of a bottleneck during the migration followed by expansion of these populations. This interpretation is consistent with the argument that America was colonized by people from Asia through Beringia sometime during the Pleistocene (Torrioni et al. 1992).

HIV-1/AIDS pathogenesis

Cytokines are major host factors regulating the pathogenesis of HIV-1 infection (Cohen et al. 1997). *IL-4* down-regulates the major HIV-1 coreceptor *CCR5*, in addition to inhibiting viral entry, replication, and cytopathogenicity (Wang et al. 1998). Five SNPs were evaluated for their potential influences on HIV-1 trans-

mission and AIDS progression, including the $-589T$ variant previously reported to associated with AIDS (Nakayama et al. 2000, 2002). Since $-589T$ upregulates the expression of *IL-4*, individuals with this allele might be less susceptible to infection. Our examination of HREU homosexual and hemophiliac European Americans, and IV-drug-using African Americans failed to identify significant associations with this SNP. However, a significantly lower frequency was reported among participants in a cross-sectional Japanese study at risk for infection through heterosexual contact, but not among hemophiliacs or homosexuals (Nakayama et al. 2000). The differences between the two studies may result from variation in study design or genetic heterogeneity among the three populations examined. *IL-4* increases the expression of *CXCR4* in $CD4^+$ primary T lymphocytes and stimulates viral expression by transcriptional activation (Valentin et al. 1998). Since *CXCR4* is the primary coreceptor used by late-stage SI viruses, one might expect the dependence of disease progression upon polymorphisms that increase *IL-4* production, such as $-589T$. Our longitudinal European American results report a significant association with $-33T$ (which is closely linked to $-589T$ on haplotype *H6*, Fig. 1) and an increased rate of progression to death, however this result is not significant after correction for multiple comparisons. Further, significance was not observed to other endpoints or in African Americans. Accordingly, we are reluctant to assign much importance to the $-33T$ association with AIDS-related death. We interpret our results as not confirming two earlier, conflicting studies where $-589T$ was associated with significantly more rapid rates of SI virus acquisition and increased serum IgE levels among infected Japanese people (Nakayama et al. 2000), and with decreased rates of progression to AIDS and death in a French cohort (Nakayama et al. 2002).

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