Polymorphisms in *IL10* may alter CD4 T-cell counts in Indonesian HIV patients beginning antiretroviral therapy

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Abbreviations

ART: Antiretroviral therapy, IL: Interleukin, SNP: Single nucleotide polymorphism

Abstract

Interleukin 10 (IL-10) is a potent anti-inflammatory cytokine influenced by single nucleotide polymorphisms (SNP) located in upstream regulatory regions. Here we address the effects of five SNP (rs1518111, rs3021094, rs3024491, rs1800872 and rs1800871) on CD4 T-cell counts in Indonesian HIV patients assessed before ART and after 12 months on treatment. Heterozygosity at rs1518111 or rs1800872 associated with low CD4 T-cell counts at both time points. Both alleles were carried in two haplotypes. Haplotype **2**11**2**2 (present in 30% of participants) associated with low CD4 T-cell counts, whereas **2**12**2**2 (in 6% of participants) did not. Hence untyped SNP(s) tagged by **2**11**2**2 may depress CD4 T-cell counts. The association with heterozygosity suggests synergy with an allele from a haplotype lacking rs1518111 and/or rs1800872.

(120 words)

1. Introduction

CD4 T-cell counts are a reliable prognostic marker of HIV disease progression and risk of opportunistic infections. HIV-infected individuals differ in their rate of disease progression and their response to antiretroviral therapy (ART). This suggests complex interactions between the virus, the environment and the host genome. Host genes known to affect HIV disease include those encoding chemokine receptors, human leukocyte antigens (HLA), cytokines and apoptosis-related genes (1, 2).

Interleukin-10 (IL-10) is an anti-inflammatory and immunomodulatory cytokine produced by macrophages, monocytes, T-helper cells and B-lymphocytes. IL-10 can inhibit the production of pro-inflammatory cytokines and down-regulate the expression of HLA class I and II molecules. IL-10 production in humans is influenced by genetic factors, which consequently have potential to affect HIV disease. Previous studies have focused on single nucleotide polymorphisms (SNP) within the *IL10* promoter region, notably -1082G/A (rs1800896), -592C/A (rs1800872) and -819C/T (rs1800871), and generate conflicting results. For example; the A allele of rs1800872 and the "ATA" haplotype defined by the three SNP increased susceptibility to HIV infection and disease progression in Indian cohorts (3,4). However in an African population, carriers of AA at rs1800872 had attenuated CD4 T-cell loss and a broader CD8 T-cell response to HIV peptides, whilst rs1800896 more clearly affected plasma IL-10 levels (5). Similarly the A alleles were protective against HIV and Hepatitis B disease in an Estonian population using intravenous drugs (6). Studies from the Indian sub-continent associate -1082G/A (rs1800896) and -592C/A (rs1800872) with susceptibility to tuberculosis amongst HIV patients, but results included associations with heterozygous carriage (7).

This highlights the need for further studies to identify *IL10* SNP that may affect CD4 T-cell counts before and on ART. A study of 21 SNP spanning *IL10* in Caucasians HIV patients confirmed the -1082G/A (rs1800896), -592C/A (rs1800872) and -819C/T (rs1800871) haplotype as the most informative marker of disease progression but showed that it tagged and its effects were modified by broader *IL10* haplotypes. Interestingly, no clear associations were found in African Americans (8).

The patterns may also be distinct in Asians, so we have investigated associations between five polymorphisms in *IL10* (rs1518111, rs3021094, rs3024491, rs1800872 and rs1800871) and CD4 T-cell count recovery in HIV-infected Indonesian patients beginning ART. We sought the haplotypes that best define the risk.

2. Materials and Methods

2.1 Patients and routine assessments: A longitudinal study of 79 HIV-1 patients was conducted at the HIV clinic at Cipto Mangunkusumo Hospital in Jakarta, Indonesia. Patients were invited to participate if they began ART with <200 CD4 T-cells/uL between March 2013 and March 2014. They were tested serologically for HCV, for oral candidiasis by clinical examination and for pulmonary tuberculosis by chest X-ray and sputum acid bacilli smear. The study was approved by the Ethics Committee of Cipto Mangunkusumo Hospital and Universitas Indonesia. Written informed consent was obtained from individuals who agreed to participate. CD4 T-cells were quantified by standard flow cytometry from the commencement of triple therapy (V0) including lamivudine, zidovudine, nevirapine, stavudine, efavirens and/or tenofovir. Most individuals were re-tested at 3, 6 and 12 months (V3, V6, V12). Reasons for discontinuation included death from causes related to AIDS (5 patients), drug-induced allergy requiring discontinuation of ART and loss to follow up. Plasma HIV RNA loads were determined using COBAS® AmpliPrep/COBAS® TaqMan® HIV-1 Tests (version 2.0).

2.2 Genotyping: DNA was quantified using a Qubit fluorometer (Thermo Fisher Scientific, Waltham, MA) and diluted to 50ng/uL. *IL10* polymorphisms (rs1518111, rs3021094, rs3024491, rs1800872 and rs1800871) were genotyped using custom TaqMan OpenArray Genotyping Plates (Life Technologies, Grand Island, NY) (9). DNA samples were diluted at 1:1 in TaqMan OpenArray Genotyping Master Mix for 50 cycles of PCR amplification. The data was analyzed using the OpenArray SNP Genotyping Analysis software, and the genotypes were assigned manually. Chi Square analysis with 5% significance level and 1 degree of freedom was used to determine if the observed allele frequencies were in Hardy-Weinberg equilibrium (HWE). Haplotypes and their estimated frequencies were calculated using the fastPHASE algorithm with the default parameters and sampled from the observed genotypes 10,000 times (10). Haplotypes with an estimated frequency <1% were excluded.

2.3 Statistical Analyses: Statistical analyses were done with GraphPad Prism software (v6; Tree Star, La Jolla, CA), using non-parametric Mann-Whitney tests, Chi^2 or Fisher's exact tests. A 5% significance level ($P < 0.05$) was considered statistically significant.

3. Results and Discussion

Seventy-nine Indonesian HIV-infected patients were screened for *IL10* polymorphisms and their effect on CD4 T-cell counts before ART and after 12 months on treatment. The cohort included 51(65%) males and had a median (range) age of 31(19-48) years. The CD4 T-cell count at baseline was 61(3-199) cells/ul. 37(47%) of the patients had pulmonary tuberculosis and 17(22%) were co-infected with Hepatitis C.

Heterosexual transmission accounted for 46 subjects (58%), with homosexual transmission and intravenous drug use each accounting for 18%. This is a common pattern in many Asian centres.
No genotypes associated with pulmonary tuberculosis, candidiasis or HCV (data not shown). Table 1 summarises associations between the five *IL10* SNP genotypes and CD4 T-cell counts. As the *IL10* rs3024491 (2,2) genotype was rare, patients with this genotype were analysed with those carrying rs3024491 (1,2). Univariate analyses associated heterozygous carriage of the minor alleles of two SNP with CD4 T-cell counts recorded before ART (rs1518111; p=0.02 and rs1800872; p=0.03) and after 3, 6 and 12 months (see Table 1), so the effect remains evident on ART. rs1518111 and rs1800872 are in linkage disequilibrium (1000 Genomes, D'=1, R^2=0.925) in East Asians and Europeans, so common associations are plausible.

The G allele of the commonly studied -1082G/A (rs1800896) was also checked and found to be rare in our Indonesian cohort (MAF=0.09). Moreover it is not in linkage disequilibrium with rs1800872 in East Asians described in the 1000 genomes database. Accordingly, carriage of the minor (G) allele of rs1800896 did not affect CD4 T-cell counts before ART or after 12 months (data not shown, p=0.77-0.83).

To resolve the effect of heterozygous carriage of rs1518111 and rs1800872 and address which SNP were responsible for the phenotype, we considered the haplotypes carried by individuals in the cohort. Fifteen haplotypes of the five *IL10* SNP described in Table 1 were derived. Five haplotypes occurred at an estimated frequency of 1% or greater and accounted for 94% of this population (Table 2). The alleles of the SNP in each haplotype are expressed as a 1 (major allele) or a 2 (minor allele), in chromosomal order as listed in Table 1. Carriage of the haplotype (**2** 1 1 **2** 2) was associated with low CD4 T-cell counts (p=0.02, Table 2). This haplotype contains the minor alleles of three SNP, where the first (rs1518111) and fourth (rs1800872) were associated with CD4 T-cell counts (Table 1). These two minor alleles also occurred in a rarer haplotype (**2** 1 2 **2** 2) that showed no association with low CD4 T-cell counts. This suggests that neither SNP is directly responsible for the low CD4 T-cell counts – rather the effect appears to reside with an unknown SNP tagged by the (**2** 1 1 **2** 2) haplotype.

In conclusion; our data links heterozygosity at *IL10* rs1518111 and rs1800872 with low CD4 T-cell counts in untreated HIV-infected Indonesian patients and on ART. Derivation of 5-SNP haplotypes demonstrated that these SNP are not directly responsible for the effect on CD4 T-cell counts. Moreover the consistent association with heterozygosity suggests synergy between SNP carried in distinct haplotypes, perhaps including the distal promoter region (8). As data in the 1000 Genomes database show distinct *IL10* haplotypes carried by different ethnic groups, studies of CD4 T-cell recovery in other ethnicities may reveal which SNP are critical. As IL-10 is critical for limiting inflammation and the host immune response to pathogens, the association with *IL10* haplotypes confirms that these pathways influence CD4 T-cell loss and recovery on ART.

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Table 1: Two *IL10* SNPs associated with low CD4 T-cell counts before and on ART.

SNP ID	V0/V3 V6/V12[a]	CD4 T-cell counts[b]			P-value[c]		
		1,1	1,2	2,2	1,1 vs 1,2	1,1 vs 2,2	1,2 vs 2,2
rs1518111 C (0.40)[d]	V0	72(9-199) (n=30)	48(3-191) (n=30)	74(2-187) (n=15)	0.02	0.18	0.91
	V3	212(79-601) (n=28)	169(7-492) (n=25)	148(11-410) (n=14)	0.08	0.04	0.50
	V6	226(118-516) (n=26)	173(6-501) (n=23)	221(20-394) (n=13)	0.04	0.31	0.83
	V12	304(171-763) (n=24)	242(121-616) (n=21)	328.5(44-736) (n=12)	0.057	0.72	0.67
rs3021094[e] G (0.46)	V0	49(2-187) (n=24)	65(3-198) (n=26)	69(9-199) (n=19)	0.72	0.12	0.26
	V12	283.5(44-736) (n=18)	285.5(121-603) (n=20)	304(171-763) (n=16)	0.87	0.5	0.32
rs3024491[f] A (0.06)	V0	62(2-199) (n=68)	94(4-191) (n=8)	-	0.49	-	-
	V12	282(44-763) (n=53)	377(101-496) (n=6)	-	0.38	-	-
rs1800872 G (0.42)	V0	69(9-199) (n=29)	45(3-191) (n=32)	52(2-187) (n=16)	0.03	0.1	0.83
	V3	206(79-601) (n=27)	136(7-492) (n=27)	159(11-410) (n=15)	0.03	0.08	0.80
	V6	242(118-516) (n=25)	157(6-501) (n=25)	226(20-394) (n=14)	0.02	0.45	0.39
	V12	304(171-763) (n=23)	207(121-616) (n=23)	298(44-736) (n=13)	0.01	0.67	0.41
rs1800871 G (0.43)	V0	67(2-187) (n=16)	48(3-198) (n=29)	67(9-196) (n=26)	0.21	0.26	0.82
	V12	286.5(144-763) (n=22)	233.5(121-616) (n=22)	328.5(44-736) (n=12)	0.21	0.96	0.79

a. V0: CD4 T-cell counts before ART. V3, V6 and V12: CD4 T-cell counts after 3, 6 and 12 months on ART
b. Median (range) CD4 T-cell counts (cells/ul)
c. Non-parametric Mann-Whitney test analysing association between the *IL10* genotypes and CD4 T-cell counts (P<0.05)
d. Minor allele (MAF), where MAF are calculated from samples genotyped successfully
e. Observed allele frequencies deviated from the expectations of HWE ((X^2 4.01 > X^2 0.05, 1 *df* (3.84))
f. (1,2) and (2,2) were merged as <5 individuals carried the (2,2) genotype

Table 2: One haplotype associated with low CD4 T-cell counts before and on ART

Haplotype Sequence[a]	V0/V12	CD4 T-cell counts[c]			P-value[d]		
		0	**1**	**2**	**1 vs 2**	**0 vs 2**	**0 vs 1**
12111 (0.41)[b]	V0	50(2-198) (n=31)	62(3-196) (n=27)	69(9-199) (n=21)	0.17	0.12	0.81
	V12	253(44-736) (n=24)	289(121-567) (n=20)	304(171-763) (n=17)	0.46	0.21	0.75
21122 (0.3)	V0	72(7-199) (n=38)	48(3-180) (n=30)	50(2-166) (n=11)	0.72	0.07	**0.02**
	V12	310(144-763) (n=29)	207(101-616) (n=23)	285(44-736) (n=9)	0.48	0.61	**0.003**
11111 (0.14)	V0	63(2-199) (n=62)	55(8-198) (n=17)	-	-	-	0.61
	V12	288(44-763) (n=49)	276(141-616) (n=12)	-	-	-	1.0
21222 (0.06)	V0	55(2-199) (n=71)	94(4-191) (n=8)	-	-	-	0.44
	V12	282(44-763) (n=55)	377(101-496) (n=6)	-	-	-	0.35
12112 (0.3)	V0	55(2-199) (n=75)	144(21-198) (n=4)	-	-	-	0.17
	V12	285(44-763) (n=58)	441(144-603) (n=3)	-	-	-	0.50

a. Defined by SNP alleles in the order shown in Table 1
b. Haplotype frequencies determined using the most probable assignment for each individual
c. Median (range) CD4 T-cells/ul. 0, 1 and 2 represent patients without the haplotype or with 1 or 2 copies
d. Non-parametric Mann-Whitney test