Frequencies of *IL10* SNP genotypes by multiplex PCR-SSP and their association with viral load and CD4 counts in HIV-1-infected Thais

Doungnapa Kingkeow[1,2], Janet M. McNicholl[3], Niwat Maneekarn[1], Jeerang Wongtrakul[2], Sineenart Taechareonkul[2], Vinai Suriyanon[2], Kenrad E. Nelson[4], Ann Duerr[3,5] and Sanit Makonkawkeyoon[1]

Summary

Background: **Interleukin (IL)-10 is an immuno-regulatory cytokine, levels of which can be influenced by single nucleotide polymorphisms (SNPs) in the promoter. Some, but not all previous studies have shown associations of *IL10* SNPs with HIV-1 disease progression, using markers such as viral load or CD4 count. There are few data on *IL10* SNP frequencies and HIV-1 disease in regions where non-B HIV-1 subtypes predominate.**

Objective: **To determine genotypes, haplotypes, allele frequencies and associations with markers of HIV-1 disease progression of *IL10* SNPs.**

Methods: **A new multiplexed PCR-SSP assay to detect *IL10* SNPs at positions -1082, -819 and -592 was developed and used to determine genotypes and haplotypes in 244 HIV-1 CRF01_AE-infected northern Thais having a median time since HIV-1 infection of 2.7 years.**

Results: **At position -1082 of *IL10*, AA genotype and A allele were the most common (87.3% and 93.2%, respectively). The -819 CT and -592 CA genotypes were the most prevalent (44.3%), and -819T and -592A were the most prevalent alleles (64.8%). The ATA/ATA was the most common genotype (42.6%) with the most prevalent haplotype of ATA (64.7%). No associations of any of the three *IL10* SNPs with CD4+ or CD8+ T cell counts or with viral load were found.**

Conclusions: **This first report of *IL10*-1082A, -819T and the *IL10*-592A allele frequencies in HIV-1-infected Thais shows the highest frequencies in HIV-1-infected persons worldwide. The lack of association of *IL10* SNPs with CD4+ T cell count and viral load suggest that other genes may influence these markers in HIV-1-infected Thais. *(Asian Pac J Allergy Immunol 2011;29:94-101)***

Key words: *HIV-1, IL10 SNPs, IL10 haplotype, multiplex PCR-SSP, viral load, CD4+ T cell*

Introduction

Interleukin (IL)-10 is an immunoregulatory cytokine that can inhibit secretion of interferon-γ (IFN-γ) and IL-2 from T cells and of tumor necrosis factor-α (TNF-α), IL-1, IL-6 and IL-8 from monocytes and macrophages. Because of this inhibitory effect on cytokine production from Th1 cells, it is generally classified as a Th2 or anti-inflammatory cytokine. It can down-regulate expression of major histocompatibility (MHC) class I on target cell surfaces, resulting in decreased cytotoxic T lymphocyte (CTL)-mediated lysis[1] and down-regulate MHC class II expression on monocytes leading to reduced antigen presentation and T cell proliferation.[2] However, IL-10 is known to stimulate B cell proliferation and promote B cell differentiation.[3]

Because of these effects, there have been many *in vitro* and *in vivo* studies of the effects of IL-10 on the pathogenesis of infectious diseases. IL-10 has been associated with pathogen persistence and poor clinical outcome of several infectious diseases including human

From the [1]Department of Microbiology
[2]Research Institute for Health Sciences, Chiang Mai University, Chiang Mai, Thailand
[3]Centers for Disease Control and Prevention, Atlanta, Georgia, USA
[4]Department of Epidemiology, John Hopkins Bloomberg School of Public Health, Baltimore, Maryland, USA
[5]HIV Vaccine Trials Center, Fred Hutchinson Cancer Research Center, University of Washington, Seattle, Washington, USA
Corresponding author: Doungnapa Kingkeow
Email: doungnapa@rihes.org
Submitted date: 20/9/2010
Accepted date: 29/12/2010

immunodeficiency virus (HIV)-1.[4] Clinically the concentration of IL-10 in patients with acquired immune deficiency syndrome (AIDS) was reported to be significantly higher than in asymptomatic HIV-1-infected patients.[5] HIV-1-infected patients with CD4+ T cell counts less than 200 cells/µl had higher frequencies of Gag-specific IL-10+ CD8+ T cells than those with CD4+ T cell counts above 200 cells/µl. These cells significantly positively correlated with plasma HIV-1 RNA[6] and suppressed IL-2 production and cytolysis by CD8+ T cells.[7] However, the role of IL-10 in HIV-1 replication *in vitro* is still controversial. Negative,[8] positive[9] or mixed effects on HIV-1 transcription or replication possibly depend on the concentration of other cytokines,[10] have been observed.

IL-10 production and transcription are, in part, regulated by *IL10* gene variation. Associations between polymorphisms at the bi-allelic *IL10* -1082 (rs1800896), -819 (rs1800871) and -592 (rs1800872) single nucleotide polymorphisms (SNPs) and levels of IL-10 production have been demonstrated.[11,12] One investigators noted that high IL-10 production was associated with *IL10* -1082G-containing genotypes[11] whereas another noted the association with *IL10*-1082A-containing genotypes.[12] The effects of different stimuli and other haplotypic associations may account for these differences. These observations led to studies of how *IL10* promoter SNPs could influence HIV-1 progression. A general conclusion of these studies was that genotypes or haplotypes containing G at position -1082 and C at position -819 and -592 had a modest beneficial effect on progression to AIDS or survival, especially in the late stages following infection.[13-15] One study in South Africa found that individuals with the *IL10* -592CC and CA genotypes had lower viral loads during the early chronic phase of infection.[16] No data are available on *IL10* promoter allele frequencies in Thais, nor are there data on how *IL10* promoter allele variation may influence markers of HIV-1 disease progression in Thais. This is particularly important since HIV-1 disease progression in persons infected with a circulating recombinant form (CRF) of HIV-1, CRF01_AE, the predominant strain in Thailand, has been reported to be more rapid than in persons infected with subtype B and several other non-B subtypes.[17] We therefore examined associations of *IL10* promoter SNPs with viral load, CD4+ and CD8+ T counts in HIV-1-infected Thais from northern Thailand where the epidemic is dominated by CRF01_AE.[18] SNP typing was performed using a multiplex polymerase chain reaction-sequence-specific primer (PCR-SSP) assay for the *IL10* -1082, -819 and -592 SNPs.

Methods

Subjects

This study included samples and data from 244 HIV-1-positive males and their female partners from several related studies.[17] Between 1989 and 1998, 590 HIV-1-seropositive men and their female partners were enrolled to determine factors affecting heterosexual transmission of HIV-1 infection in Northern Thailand (also termed the parent or CONRAD study). Subjects were identified through blood donation at the blood bank of Chiang Mai University Hospital, Chiang Mai Thai Red Cross or the Regional Hospital in Lampang. In 1999, a subsequent survival study (also known as SSI) took place in which survival of these 590 couples was determined from questionnaires and government records, and some additional blood samples were obtained. In 2000-2002 a subset of the initial couples who were HIV-1-infected, remained alive and consented to enroll were enrolled in a study of host factors, viral factors and HIV-1 disease progression (also known as SSII). Enrollment date used in our study was enrollment date of CONRAD study. Dates of seroconversion were estimated as previously described[18] and were then used to calculate duration of infection at date of enrollment. None of the patients in the study had received any anti-retroviral therapy at the date of enrollment. All laboratory testing was performed at enrollment and in some cases at 6 and 12 months follow-up. HIV-1 antibody testing used the enzyme-linked immunosorbent Combitest (Abbot Laboratories, North Chicago, IL, USA) and was confirmed by Western blot (Dupont, Wilmington, DE, USA). CD4+ and CD8+ cell T counts were determined using the Simultest™ IMK-lymphocyte assay (Becton Dickinson, San Jose, CA, USA). HIV-1 RNA was determined using the Roche Amplicor HIV Monitor assay version 1.5 (Roche Molecular Systems, Branchburg, NJ, USA) with a lower detection limit of 50 HIV RNA copies/ml. For the purposes of analysis values <50 were assigned a value of 49.

Table 1. Primer sequences, annealing temperature and amplicon size for the single locus PCR-SSP and sequencing

Primer 5'-3'	Ta (°C)	Amplicon (bp)
Single locus PCR-SSP		
IL10-1082 common reverse CAG TGC CAA CTG AGA ATT TGG *IL10*-1082G forward CTA CTA AGG CTT CTT TGG GAG *IL10*-1082A forward ACT ACT AAG GCT TCT TTG GGA A	66.0	258
IL10-819 common reverse AGG ATG TGT TCC AGG CTC CT *IL10*-819C forward CCC TTG TACAGG TGA TGT AAC *IL10*-819T forward ACC CTT GTA CAG GTG ATG TAA T	65.0	233
IL10-592 common reverse CAA GCC CCT GAT GTG TAG A *IL10*-592C forward CTG TGA CCC CGC CTG TC *IL10*-592A forward CTG TGA CCC CGC CTG TA	66.0	600
HGH-1 GCC TTC CCA ACC ATT CCC TTA *HGH*-2 TCA CGG ATT TCT GTT GTG TTT C	62.0	429
Sequencing		
IL10 promoter forward ATC TGA AGA AGT CCT GAT GT *IL10* promoter reverse AAC TTA GGC AGT CAC CTT A	58.4	786

Abbreviation: Ta, annealing temperature

All assays were performed according to the manufacturer's instructions. Enrollment CD4+ and CD8+ T cell counts and viral load were used in this study. Viral subtype was determined using a peptide EIA as previously described.[19] At the time when genetic sub-studies were initiated, 885 persons of the 590 couples had complete data for analysis but only 244 subjects had available samples (stored peripheral blood mononuclear cells or polymorphonuclear cells) including 159 CONRAD, 33 SSI and 52 SSII study patients. There were no significant differences in age, gender, duration of infection, CD4+ and CD8+ T cell counts as well as viral load between the 244 and 885 subjects ($P > 0.05$). Additional DNA samples were obtained from 23 HIV-1-uninfected individuals working in Maharaj hospital, Chiang Mai through a separate study which was approved by the Research Ethics Committee, Faculty of Medicine, Chiang Mai University. All studies were approved by the Research Institute for Health Sciences i*nstitutional review board* (IRB) in Thailand and the three studies of HIV-1-infected persons had additional approvals from Johns Hopkins University and CDC IRBs in the US. Informed consent was obtained from all of the participating subjects. Specimens were coded with unique identifiers.

Conventional single locus PCR-SSP

To generate samples of known *IL10* SNP types, 100 DNA samples (23 from healthy HIV-1-uninfected persons and 77 from HIV-1-positive persons with abundant DNA) were selected and tested by single locus PCR-SSP at positions -1082, -819 and -592. Forward and reverse primers for -1082 and -819 were as described previously.[20] All other primers were designed by the Primer 3 program[21] using the *IL10* promoter gene sequence from Genbank, AF295024.[22] Human growth hormone (HGH) primers, as described previously,[23] were used as internal controls to check the success of PCR amplification. Two tube reactions were performed per SNP, one for the normal allele and the other for the variant allele. Amplification was performed in 10 µl with 25-50 ηg DNA sample, 20 mM Tris-HCl (pH 8.4), 50 mM KCl, 2 mM $MgCl_2$, 0.2 mM each dNTPs, 2 µM each specific/common primers (Table 1), 0.25 µM each human growth hormone (HGH) primers, and 0.25 units of Platinum®Taq DNA polymerase (Invitrogen™ life technologies, Sao Paulo, Brazil). Cycling was conducted using a Tetrad DNA engine (MJ Research, Inc., Watertown, MA, USA) or a 9700 thermocycler (Appiled Biosystems, Foster City, CA, USA). The reaction

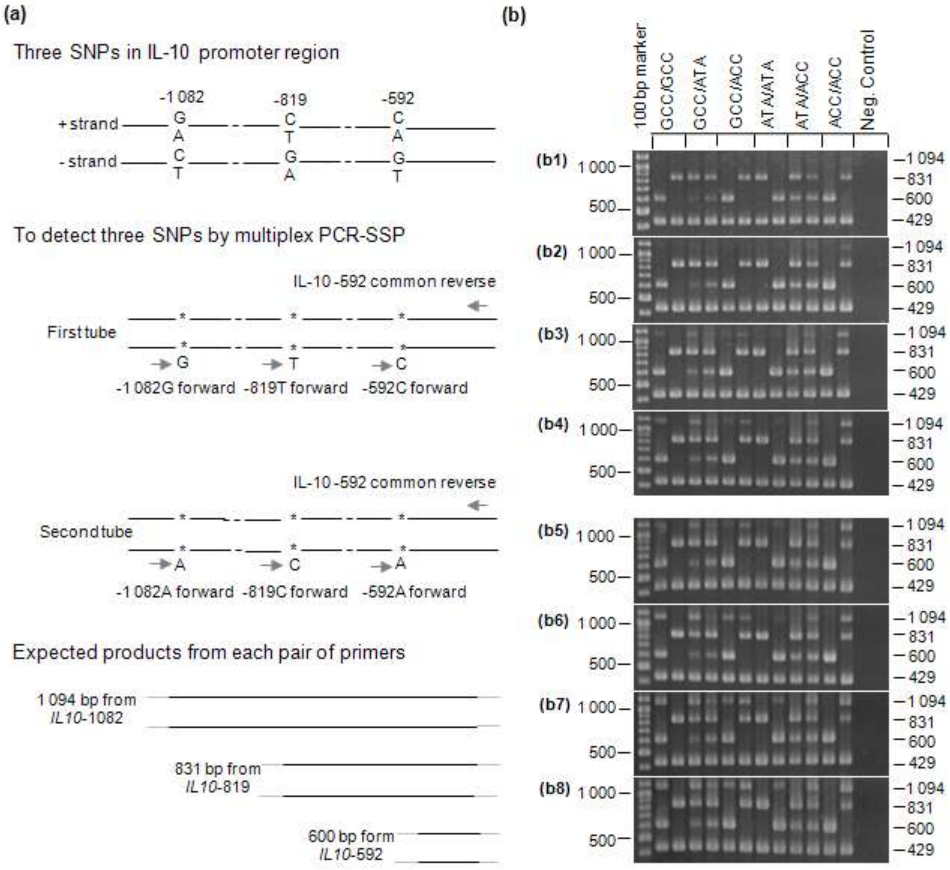

Figure 1. Schematic of the multiplex PCR-SSP method (a) and the condition set up of the multiplex PCR-SSP (b) with various cycle number of 20 (b1), 25 (b2), 30 (b3) and 35 (b4) and various extension time of 50 s (b5), 60 s (b6), 70 s (b7) and 80 s (b8). In all gel, lane 2, 4, 6, 8, 10 and 12 are PCR product from the first-tube reaction and lane 3, 5, 7, 9, 11 and 13 are those from the second-tube reaction. Each lane includes an internal PCR control amplicon of human growth hormone gene (429 bp).

mixture was initially heated at 94°C for 2 min, and DNA amplification was done using 20 cycles of 95°C for 20 s, Ta for each SNP (Table 1) for 50 s, 72°C for 50 s, and to amplify HGH, 10 cycles of 95°C for 15 s, 62°C for 50 s, and 72°C for 40 s. The genotype of each SNP was determined by the length of the amplicon (Table 1) after gel electrophoresis on 2% agarose gel and then stained with 0.5 µg/ml ethidium bromide. Haplotypes detected using this method were assigned as GCC, ACC or ATA. DNA from 25 samples with the likely genotypes of GCC/GCC, GCC/ACC, GCC/ATA, ACC/ACC, ACC/ATA, ATA/ATA, were selected for sequencing to confirm the SNPs. PCR for sequencing was performed in a 50 µl reaction mixture. The amplification conditions were the same as for conventional PCR-SSP except 0.2 µM each *IL10* promoter forward and reverse primers (Table 1). The reaction was heated at 94°C for 2 min, followed by 40 cycles of 95°C for 30 s, 58.4°C for 30 s, 72°C for 1 min, and at 72°C for 5 min at the end of cycle 40. The amplified DNA fragments were purified by QIAquick PCR Purification Kit (Qiagen, Hilden, Germany) and sequenced by *Macrogen* Inc. (Seoul, *Korea*) using the Applied Biosystems Sequencer Model 3730XL (Applied Biosystems). Samples with known genotypes were then used to set up the multiplex PCR-SSP and as positive controls for genotyping all remaining assays.

Multiplex PCR-SSP

The primers, PCR mixture and electrophoresis conditions were as described for single locus PCR-SSP. The assay used a two-tube reaction per sample (Figure 1a). One reaction contained primer mixtures of *IL10*-1082G forward, *IL10*-819T forward, *IL10*-592C forward and *IL10*-592 common reverse. The other contained primer mixtures of *IL10*-1082A forward, *IL10*-819C

forward, *IL10*-592A forward and *IL10*-592 common reverse. Optimal thermocycler conditions for amplification were evaluated including: 94°C for 2 min, 20, 25, 30 or 35 cycles of 95°C for 20 s, 66°C for 50 s, 72°C for 50 s, followed by 10 cycles of 95°C for 15 s, 62°C for 50s, and 72°C for 40 s. Then, extension times from 50, 60, 70 or 80 s were evaluated. Increasing yields of all products were obtained with increasing numbers of cycles (Figure 1b, b1-b4). Based on this data, 35 PCR cycles was selected. Since the band for the long 1,094 bp product of *IL10*-1082 SNP was still faint and difficult to observe, different extension times (Figure 1b, b5-b8) were evaluated, with 80 s extension being optimal.

Statistical analysis

Hardy-Weinberg Equilibrium (HWE) and linkage disequilibrium (LD) were determined using the program described by Rodriguez *et al*.[24] and Gaunt *et al*.[25], respectively. The t test was used to compare age and the Kruskal-Wallis one way ANOVA (SPSS, version 17.0, Chicago, IL, USA) was used to compare duration of infection, CD4+ and CD8+ T cell counts and viral load across genotypic groups.

Results

Demographic, CD4+ and CD8+ T cell counts and viral load data of enrolled subjects

Of the 244 northern Thais 158 (64.8%) were male and 86 (35.2%) were female. At enrollment, the average age was 28.7 ± 6.4 years. The median [interquartile range (IQR)] duration of infection was 2.7 years (1.3-3.4 years). Baseline CD4+ and CD8+ T cell counts were available in 241 individuals. Median CD4+ and CD8+ T cell counts were 371 cells/µl (224-558 cells/µl) and 965 cells/µl (714-1,396 cells/µl). Of the 237 samples with available viral load data, only 3 were undetectable and median viral load was 4.3 $\log_{10}$ copies/ml (3.8-4.8 $\log_{10}$ copies/ml).

Table 2. *IL10* genotype, allele and haplotype frequencies in 244 HIV-1-positive individuals

Genotype	No. (%)	Allele and haplotype	No. (%)
IL10-1082			
GG	2 (0.8)	G	33 (6.8)
GA	29 (11.9)	A	455 (93.2)
AA	213 (87.3)		
IL10-592 (-819)			
CC	32 (13.1)	C	172 (35.2)
CA(CT)	108 (44.3)	A (T)	316 (64.8)
AA(TT)	104 (42.6)		
IL10-1082, -819, -592			
GCC/GCC	2 (0.8)	Haplotype GCC	33 (6.8)
GCC/ACC	7 (2.9)	Haplotype ACC	139 (28.5)
GCC/ATA	22 (9.0)	Haplotype ATA	316 (64.7)
ACC/ACC	23 (9.4)		
ACC/ATA	86 (35.3)		
ATA/ATA	104 (42.6)		

Multiplex PCR-SSP genotyping of IL10 promoter SNPs in HIV-1-infected Thais

The multiplex assay correctly identified 23 normal and 77 HIV-1-infected samples previously typed by single locus PCR-SSP and gave the same results as the 25 samples which were typed by sequencing indicating that it was accurate and specific.

The remaining HIV-1-infected Thais were also tested by multiplex PCR-SSP. *IL10* SNP data for the 244 HIV-1-infected Thais are presented in Table 2. The genotype distributions of the *IL10* -1082, -819 and -592 SNPs were in HWE (*P*= 0.67, 0.90 and 0.90, respectively). At position *IL10*-1082, the AA genotype was the most common (87.3% in HIV-1 positives) and A was the most prevalent allele, found in more than 90% of persons. The *IL10*-592C and -592A alleles were in complete linkage disequilibrium with -819C and -819T, respectively (D´ = 1.0, *P* = 0.000). The -819 CT and -592 CA genotypes were the most prevalent (44.3% in HIV-1 positives) and -819T and -592A were the most prevalent alleles (each 64.8%). Homozygosity for the ATA haplotype was found in 42.6% of HIV-1 positives and the most prevalent haplotype was ATA (64.7%) followed by ACC (28.5%).

Table 3. Characteristics of HIV-1-infected individuals at enrollment by *IL10*-1082 genotype

Characteristics	GG or GA (n=31)	AA (n=213)	*P*-value
Age, mean years (SD)	27.9 (5.3)	28.9 (6.6)	0.461
Duration of infection, median days (IQR)	2.1 (0.1-3.3)	2.7 (1.4-3.4)	0.138
CD4+ T cell count, median cells/µl (IQR)[a]	441 (286-594)	353 (222-547)	0.298
CD8+ T cell count, median cells/µl (IQR)[a]	1,068 (741-1,398)	960 (710-1,397)	0.715
HIV load, median $\log_{10}$ HIV RNA copies/ml (IQR)[b]	4.3 (3.8-4.8)	4.3 (3.8-4.8)	0.708

[a] Number available for 30 and 211 subjects, respectively

[b] Number available for 29 and 208 subjects, respectively

Associations of IL10 *promoter SNPs with CD4+ and CD8+ T cell counts and viral load in HIV-1-infected Thais*

Because of the influence of *IL10* SNPs on IL-10 production and their previous associations with HIV-1 diseases progression or viral load levels we compared CD4+ and CD8+ T cell counts and viral loads in HIV-1-infected subjects with different *IL10* genotypes. At position -1082, because of the rarity of the *IL10*-1082 GG genotype, subjects with this genotype were combined with those with the *IL10*-1082 GA genotype and compared to those with the AA genotype (Table 3). No significant differences in CD4+, CD8+ T cell counts or viral load were seen in this analysis.

The *IL*10-592 and -819 SNPs are in linkage disequilibrium and therefore we present only the *IL10*-592 genotype data. At position -592 (Table 4), no differences in CD4+, CD8+ T cell counts or viral load were seen between subjects with each genotypes. Since all variables in subjects with CA and AA genotype were similar (P > 0.05, data not shown), data from persons with these genotypes were combined together. There were no significant associations between -592 SNP genotypes and any parameter studied (Table4).

Table 4. Characteristics of HIV-1-infected individuals at enrollment by *IL10*-592 genotype

Characteristics	CC* (n=32)	CA * (n=108)	AA * (n = 104)	CA or AA * (n=212)
Age, mean years (SD)	29.3 (6.6)	28.8 (6.6)	28.4 (6.3)	28.7 (6.6)
Duration of infection, median days (IQR)	2.4 (0.9-3.4)	2.6 (1.4-3.4)	2.8 (1.3-3.4)	2.7 (1.4-3.4)
CD4+ T cell count, median cells/µl (IQR)[a]	372 (243-515)	370 (194-591)	372 (224-555)	370 (222-578)
CD8+ T cell count, median cells/µl (IQR)[a]	978 (804-1,380)	1 037 (693-1,442)	928 (711-1,355)	965 (704-1,400)
HIV load, median $\log_{10}$ HIV RNA copies/ml (IQR)[b]	4.6 (4.0-5.0)	4.3 (3.7-4.7)	4.3 (3.8-4.8)	4.3 (3.7-4.8)

[a] Number available for 32, 107, 102 and 209 subjects, respectively
[b] Number available for 31, 104, 102 and 206 subjects, respectively
**P*-values for all comparison of CC group vs CA group, CC group vs AA group, CA group vs AA group or CC group vs combined (CA or AA) groups > 0.05. The viral load comparisons of CC vs CA/AA groups were borderline significant (P = 0.057)

However, there was a non-significant trend for lower viral loads in persons with the CA or AA genotype compared to those with CC genotypes (4.3 vs 4.6 $\log_{10}$ copies/ml, P = 0.057).

Discussion

A multiplex PCR-SSP assay was established that can detect the three neighboring bi-allelic *IL10* promoter polymorphisms at position -1082, -819 and -592. The assay generates amplicons of different lengths that can be easily differentiated by gel electrophoresis. Although more rapid typing platforms are available using expensive equipment, this assay uses simple equipment, with increased throughput and reduced costs compared to single locus PCR-SSP. The method could be adapted to other SNPs relevant for evaluating immune responses where the positions to be tested are in close proximity such as: *RANTES*-28 and -403; *IL1β*-511 and -31 and; *IL4*-33, -590 and -1098.

Using this method, we report, for the first time, the frequencies of *IL10* SNP genotypes in Thais, particularly among HIV-1-infected northern Thais who were enrolled and became HIV-1 infected in the late 1980s and mid-1990s during the early part of the CRF01_AE epidemic in Thailand. Infection was primarily heterosexual. Although the subjects enrolled had been infected for up to 3.4 years, CD4+ T cell counts were relatively preserved in most, with a median of 371 cells/µl. A striking observation is the very high frequency (93.2%) of *IL10*-1082A in HIV-1-infected northern Thais. This is the highest reported frequency of this allele in any HIV-1-infected population. A study of HIV-1-infected northern Indians reported a frequency of -1082A of 72.2 %.[14] Slightly lower frequencies were reported in studies of Africans or African-Americans, with a frequency of 70% in a Zimbabwean study of HIV-1-infected persons,[15] 67% in a South African study of HIV-1-infected and HIV-1-negative persons[16] and 64.3% in a study of HIV-1-positive African Americans.[26] A much lower frequency was reported in HIV-1-infected Hispanics in North America (38.8 %).[26] Although lower in frequency than -1082A, most (64.8%) HIV-1-infected northern Thais were *IL10* -592A and -819T positive, a prevalence higher than reported in HIV-1-infected persons in northern India (42.2%),[14] some African countries (40.5%),[15] African Americans (38.2 %)[26] and in a South African study of HIV-1-infected and HIV-

1-negative persons (32.0%).[16] These are more than double the frequencies reported in HIV-1-positive North American Hispanics (28.2 %)[26] and Caucasians (24-25 %).[27] Most (64.7%) HIV-1-infected northern Thais were positive for the *IL10*-1082, -819, -592 ATA haplotype. The frequency of this haplotype is higher than in other populations: 42.2% in a study of HIV-1-positive northern India;[14] 44% in an African American study[28] and 21% in a study of HIV-1-infected and uninfected European Americans.[28]

The trend toward a higher prevalence of *IL10*-1082A, -819T and -592A alleles and of the *IL10* ATA haplotype in HIV-1-infected Asian populations compared to others likely reflects general population frequencies of these alleles and the haplotype. Supportive of this are the high prevalence of these alleles (95.6, 63.0 and 63.0% for -1082A, -819T and -592A, respectively) and the ATA haplotype (63.0%) in the small number of uninfected northern Thais recruited for the purposes of assay optimization in this study. In addition, the prevalence of -1082 AA genotypes is known to be extremely high in some other Asian populations (e.g. 98.0% in Taiwanese and 95.1% in Singapore Chinese) and the lowest in Caucasians (e.g. 7.0% in Italians)[29] suggesting an east to west cline in the distribution of certain *IL10* alleles. Within Thailand, there may even be regional variation at the *IL10* locus since previous studies have shown that northern Thais have different prevalences of HLA genes, such as DRB1*07011/2, than Thais in other parts of the country.[30,31]

In our sero-prevalent subjects with a median duration of infection of 2.7 years we found no association between *IL10*-1082 SNP and baseline viral load. This is similar to findings from a recent study in South Africa where the -1082 GG genotype (predicted to be associated with high IL-10 production in some studies) was not associated with viral loads during the early chronic phase of HIV-1 infection.[16]

The South African study found that the -592 AA genotype (predicted to be associated with low IL-10 production in some studies) was associated with higher viral loads in the early chronic phase of HIV-1 infection.[16] In our study, we found no association of the -592A-containing genotypes and viral load in northern Thais although there was a non-significant trend toward lower viral loads in northern Thais with the -592A-containing genotypes. Differences for these observations may reflect differences in sample size (244 subjects in our study vs 64 in the S. African study), duration of infection at time of analysis (2.7 years in our study vs 6 months in the S. African study), infecting subtype (CRF01_AE vs subtype C), haplotypic or other genetic factors, or other factors unrelated to HIV-1 or *IL10* genotypes. Some previous studies have noted associations of -592A-containing genotypes with rapid progression. A study in northern India observed more rapid progression to AIDS in persons with the -592 AA genotpyes[14] and the -592 AA or -592CA genotypes or the ATA haplotype have been associated with rapid progression in Caucasian and European Americans but not in African Americans.[13,28] While we did not examine associations with disease progression it is known that higher peak and set-point viral loads may be associated with more rapid HIV-1 disease progression. Our finding of no association of -592A-containing genotypes and viral load might predict that these genotypes might not influence progression to AIDS in northern Thais. However, key difference between northern Thais and these other populations, in particular the very high prevalence of -592A-containing genotypes may influence how other *IL10* genotypes influence viral loads or clinical outcomes in that their effects may be masked if a certain cytokine mileu predominates. These findings suggest that the same genotype or haplotype may be associated with a different phenotype in the setting of different genetic prevalences as shown in previous studies of other cytokine genes.[26,27,32-35]

In conclusion, the multiplex *IL10* PCR-SSP is simple, rapid and suitable for resource-limited laboratories. HIV-1-infected Thais were found to have the highest reported allele and haplotype frequencies of SNPs associated, in some studies, with low IL-10 production. Our study showed no significant association of any *IL10* promoter SNP and viral load or CD4 counts in HIV-1-infected northern Thais.

Acknowledgements

This work was supported by a Royal Golden Jubilee Grant of the Thailand Research Fund (TRF), Thailand Fiscal Year 2003-2005 and the National Research University Project under Thailand's Office of the Higher Education Commission. Doungnapa Kingkeow was

supported by a Royal Golden Jubilee Ph.D. Program. JHU, CDC and RIHES supported the parent studies that enrolled HIV-1-positive subjects into the cohort studies. We are grateful to staffs at the blood bank in the Chiang Mai University hospital, Thai Red Cross in Chiang Mai, Regional hospital in Lampang and RIHES for their contribution to the study. We also thank Michael Aidoo for immunogenetic training and his invaluable advice.

Conflicts of Interest Disclosure

The authors have no conflicts of interest with this work.

References

1. Matsuda M, Salazar F, Petersson M, Masucci G, Hansson J, Pisa P, et al. Interleukin 10 pretreatment protects target cells from tumor- and allo-specific cytotoxic T cells and downregulates HLA class I expression. J Exp Med. 1994;180:2371-6.
2. de Waal Malefyt R, Haanen J, Spits H, Roncarolo MG, te Velde A, Figdor C, et al. Interleukin 10 (IL-10) and viral IL-10 strongly reduce antigen-specific human T cell proliferation by diminishing the antigen-presenting capacity of monocytes via downregulation of class II major histocompatibility complex expression. J Exp Med. 1991;174:915-24.
3. Rousset F, Garcia E, Defrance T, Peronne C, Vezzio N, Hsu DH, et al. Interleukin 10 is a potent growth and differentiation factor for activated human B lymphocytes. Proc Natl Acad Sci U S A. 1992;89:1890-3.
4. Breen EC, Boscardin WJ, Detels R, Jacobson LP, Smith MW, O'Brien SJ, et al. Non-Hodgkin's B cell lymphoma in persons with acquired immunodeficiency syndrome is associated with increased serum levels of IL10, or the IL10 promoter -592 C/C genotype. Clin Immunol. 2003;109:119-29.
5. Ramalingam S, Kannangai R, Vijayakumar TS, Mathai D, Abraham OC, Subramanian S, et al. Subtype & cytokine profiles of HIV infected individuals from south India. Indian J Med Res. 2005;121:226-34.
6. Elrefaei M, Barugahare B, Ssali F, Mugyenyi P, Cao H. HIV-specific IL-10-positive CD8+ T cells are increased in advanced disease and are associated with decreased HIV-specific cytolysis. J Immunol. 2006;176:1274-80.
7. Elrefaei M, Ventura FL, Baker CA, Clark R, Bangsberg DR, Cao H. HIV-specific IL-10-positive CD8+ T cells suppress cytolysis and IL-2 production by CD8+ T cells. J Immunol. 2007;178:3265-71.
8. Akridge RE, Oyafuso LK, Reed SG. IL-10 is induced during HIV-1 infection and is capable of decreasing viral replication in human macrophages. J Immunol. 1994;153:5782-9.
9. Rabbi MF, Finnegan A, Al-Harthi L, Song S, Roebuck KA. Interleukin-10 enhances tumor necrosis factor-alpha activation of HIV-1 transcription in latently infected T cells. J Acquir Immune Defic Syndr Hum Retrovirol. 1998;19:321-31.
10. Finnegan A, Roebuck KA, Nakai BE, Gu DS, Rabbi MF, Song S, Landay AL. IL-10 cooperates with TNF-alpha to activate HIV-1 from latently and acutely infected cells of monocyte/macrophage lineage. J Immunol. 1996;156:841-51.
11. Edwards-Smith CJ, Jonsson JR, Purdie DM, Bansal A, Shorthouse C, Powell EE. Interleukin-10 promoter polymorphism predicts initial response of chronic hepatitis C to interferon alfa. Hepatology. 1999;30:526-30.
12. Eskdale J, Keijsers V, Huizinga T, Gallagher G. Microsatellite alleles and single nucleotide polymorphisms (SNP) combine to form four major haplotype families at the human interleukin-10 (IL-10) locus. Genes Immun. 1999;1:151-5.
13. Shin HD, Winkler C, Stephens JC, Bream J, Young H, Goedert JJ, et al. Genetic restriction of HIV-1 pathogenesis to AIDS by promoter alleles of IL10. Proc Natl Acad Sci U S A. 2000;97:14467-72.
14. Chatterjee A, Rathore A, Sivarama P, Yamamoto N, Dhole TN. Genetic Association of IL-10 Gene Promoter Polymorphism and HIV-1 Infection in North Indians. J Clin Immunol. 2009;29:71-7.
15. Erikstrup C, Kallestrup P, Zinyama-Gutsire RB, Gomo E, Butterworth AE, Pedersen BK, et al. Reduced mortality and CD4 cell loss among carriers of the interleukin-10 -1082G allele in a Zimbabwean cohort of HIV-1-infected adults. AIDS. 2007;21:2283-91.
16. Naicker DD, Werner L, Kormuth E, Passmore JA, Mlisana K, Karim SA, Ndung'u T. Interleukin-10 Promoter Polymorphisms Influence HIV-1 Susceptibility and Primary HIV-1 Pathogenesis. J Infect Dis. 2009;200:448-52.
17. Nelson KE, Costello C, Suriyanon V, Sennun S, Duerr A. Survival of blood donors and their spouses with HIV-1 subtype E (CRF01 A_E) infection in northern Thailand, 1992-2007. AIDS. 2007;21 Suppl 6:S47-54.
18. Costello C, Nelson KE, Suriyanon V, Sennun S, Tovanabutra S, Heilig CM, et al. HIV-1 subtype E progression among northern Thai couples: traditional and non-traditional predictors of survival. Int J Epidemiol. 2005;34:577-84.
19. Pau CP, Lee-Thomas S, Auwanit W, George JR, Ou CY, Parekh BS, et al. Highly specific V3 peptide enzyme immunoassay for serotyping HIV-1 specimens from Thailand. AIDS. 1993;7:337-40.
20. Perrey C, Turner SJ, Pravica V, Howell WM, Hutchinson IV. ARMS-PCR methodologies to determine IL-10, TNF-alpha, TNF-beta and TGF-beta 1 gene polymorphisms. Transpl Immunol. 1999;7:127-8.
21. http://frodo.wi.mit.edu/primer3/. In: Primer3; Assessed on 04/12/2007.
22. http://www.ncbi.nlm.nih.gov/Genbank/index. In: Nucleotide; Accessed on 03/07/2007.
23. Aldener-Cannava A, Olerup O. HLA-DOB1 "low-resolution' typing by PCR amplification with sequence-specific primers (PCR-SSP). Eur J Immunogenet. 1994;21:447-55.
24. Rodriguez S, Gaunt TR, Day IN. Hardy-Weinberg equilibrium testing of biological ascertainment for Mendelian randomization studies. Am J Epidemiol. 2009;169:505-14.
25. Gaunt TR, Rodriguez S, Day IN. Cubic exact solutions for the estimation of pairwise haplotype frequencies: implications for linkage disequilibrium analyses and a web tool 'CubeX'. BMC Bioinformatics. 2007;8:428.
26. Wang C, Song W, Lobashevsky E, Wilson CM, Douglas SD, Mytilineos J, et al. Cytokine and chemokine gene polymorphisms among ethnically diverse North Americans with HIV-1 infection. J Acquir Immune Defic Syndr. 2004;35:446-54.
27. Vasilescu A, Heath SC, Ivanova R, Hendel H, Do H, Mazoyer A, et al. Genomic analysis of Th1-Th2 cytokine genes in an AIDS cohort: identification of IL4 and IL10 haplotypes associated with the disease progression. Genes Immun. 2003,4:441-9.
28. Oleksyk TK, Shrestha S, Truelove AL, Goedert JJ, Donfield SM, Phair J, et al. Extended IL10 haplotypes and their association with HIV progression to AIDS. Genes Immun. 2009;10:309-22.
29. http://www.allelefrequencies.net. In: Allele Frequencies in Worldwide Populations;Accessed on 02/02/2010.
30. Chandanayingyong D, Stephens HA, Klaythong R, Sirikong M, Udee S, Longta P, et al. HLA-A, -B, -DRB1, -DQA1, and -DQB1 polymorphism in Thais. Hum Immunol. 1997;53:174-82.
31. Stephens HA, Chandanayingyong D, Kunachiwa W, Sirikong M, Longta K, Maneemaroj R, et al. A comparison of molecular HLA-DR and DQ allele profiles forming DR51-, DR52-, and DR53-related haplotypes in five ethnic Thai populations from mainland southeast Asia. Hum Immunol. 2000;61:1039-47.
32. Brinkman BM, Keet IP, Miedema F, Verweij CL, Klein MR. Polymorphisms within the human tumor necrosis factor-alpha promoter region in human immunodeficiency virus type 1-seropositive persons. J Infect Dis. 1997;175:188-90.
33. Knuchel MC, Spira TJ, Neumann AU, Xiao L, Rudolph DL, Phair J, et al. Analysis of a biallelic polymorphism in the tumor necrosis factor alpha promoter and HIV type 1 disease progression. AIDS Res Hum Retroviruses. 1998;14:305-9.
34. Nakayama EE, Hoshino Y, Xin X, Liu H, Goto M, Watanabe N, et al. Polymorphism in the interleukin-4 promoter affects acquisition of human immunodeficiency virus type 1 syncytium-inducing phenotype. J Virol. 2000;74:5452-9.
35. Wichukchinda N, Nakayama EE, Rojanawiwat A, Pathipvanich P, Auwanit W, Vongsheree S, et al. Protective effects of IL4-589T and RANTES-28G on HIV-1 disease progression in infected Thai females. AIDS. 2006;20:189-96.