ORIGINAL ARTICLE

Association of -308 TNF-alpha promoter polymorphism with viral load and CD4 T-helper cell apoptosis in HIV-1 infected black South Africans

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Objective. To determine whether the -308 TNF-α promoter polymorphism is associated with markers of HIV progression in the South African population.

Methods. Polymerase chain reaction-restriction fragment length polymorphism was used to detect the -308 TNF-α polymorphism in 75 patients and 76 healthy controls. Serum TNF-α concentrations were measured using ELISA in each cohort. $CD4^+$ T cell apoptosis and HIV-1 RNA viral load were determined using Annexin-V-FITC assay and Nuclisens Easy Q HIV-1 assay respectively. $CD4^+$ T cell counts were measured flow cytometrically.

Results. The frequency of -308 G allele was similar in the HIV-1 and control cohorts. The -308GG genotype was associated with lower TNF-α concentrations and markers of increased HIV progression indicated by higher T_H lymphocyte apoptosis, lower T_H lymphocyte count and higher plasma viral load, irrespective of treatment.

Conclusion. The presence of the TNF-α -308 G allele in HIV-1 patients may be associated with increased risk of HIV-1 progression. Further research is required to investigate the nature of this association.

S Afr J HIV Med 2012;13(2):72-77.

Patients infected with human immunodeficiency virus (HIV) show a decline in $CD4^+$ T-helper (T_H) lymphocyte levels and an increase in viral load that ultimately results in compromised immune function and increased susceptibility to various opportunistic infections.[1] In early stages of infection, HIV-1 has the ability to manipulate the immune response to ensure its own replication and survival.[2] Consequently, there has been much controversy as to whether eliciting a robust immune response towards the virus early in infection will be beneficial or detrimental for the patient.[2]

The differential rate of HIV progression and chronic inflammatory disorders[3-5] may be induced by viral, environmental and host genetic factors. Dean *et al.* observed a 32 base pair deletion in the chemokine receptor 5 (CCR5) that showed better protection against HIV and slower progression to AIDS.[6] Another study investigated a chemokine receptor 2 (CCR2) polymorphism with a G→A transition at position 190, that also resulted in slower progression to AIDS.[7] Crawley *et al.* found that a polymorphism associated with IL-10 at the -592 position resulted in decreased production of IL-10, inhibition of macrophage growth and decreased proliferation of HIV-1 in infected individuals.[8,9] The molecular mechanisms of most polymorphisms have not been fully elucidated. There is a need to explore more the role of host genetics in understanding HIV disease.

In vitro and *in vivo* studies have shown that HIV-1 infection can induce the secretion of pro-inflammatory cytokines such as tumour necrosis factor alpha (TNF-α).[10-12] TNF-α is the central mediator of the inflammatory response, and high concentrations of TNF-α may influence HIV-1 replication via clonal expansion of infected T lymphocytes.[13] In addition, TNF-α is also a potent inducer of apoptosis, which is a function dependent on the death receptor configuration of immune cells.[1,14] HIV-1 induces immune suppression by rapid apoptosis of bystander T_H lymphocytes.

TNF-α production is tightly controlled but genotypic differences may influence transcriptional regulation.[15,16] Reports have shown that promoter polymorphisms affect TNF-α gene expression.[17-19] A common polymorphism occurs at the -308 locus in the promoter region that results in

a guanine (G) to adenine (A) transition.[20] The -308 A allele has been associated with higher transcriptional activation and, therefore, increased TNF-α expression in different populations.[4,17,19] This association has also been linked to pathogenesis of various inflammatory disorders and, consequently, poorer disease prognosis.[4,17] The presence of various allelotypes, especially in promoter regions of cytokines, may severely affect immune responses to infection, given that they exert a large degree of transcriptional control over cytokine production. These effects, however, have not been comprehensively investigated in the context of infection. The precise mechanisms of genotypic influences on transcriptional regulation are currently unknown. However, it is thought that the G to A transition at the -308 locus is associated with conformational changes that increase binding affinity of transcription factors such as nuclear factor-kappa B (NF-κB).[15-17]

Considering the influence of the -308 TNF-α promoter polymorphism on TNF-α concentration, CD4 T_H lymphocyte apoptosis and HIV-1 replication, genotype may severely influence clinical outcomes in HIV-1 infected patients. The influence of the -308 TNF-α promoter polymorphism on HIV-1 infected black South Africans has not been studied. This is important as South Africa has the highest burden of HIV-1 infected individuals, and polymorphic variation may not only affect disease progression, but also response to treatment.

The aim of this study was to investigate genotypic frequencies of the -308 TNF-α promoter polymorphism in a cohort of HIV-1 infected black South African patients and determine whether genotype at this locus influenced serum TNF-α concentrations. In addition, the influence of this promoter polymorphism on CD4 T_H lymphocyte apoptosis and HIV-1 burden was investigated.

Materials and methods

Patient recruitment

This cross-sectional study was approved by the University of KwaZulu-Natal, Biomedical Research Ethics Administration (H129/04). Patients (*N*=75) were recruited by purposeful sampling from an antiretroviral (ARV) rollout clinic at King Edward VII Hospital, Durban, after obtaining informed consent. All patients had confirmed HIV-1 infection. Twenty-five patients were on NRTI-based HAART (NRTI: nucleoside reverse transcriptase inhibitor; HAART: highly active anti-retroviral therapy); 50 patients were HAART-naive. Healthy controls (*N* =76) were sourced from the South African National Blood Service. There was no follow-up of patients to assess changes in measures or outcomes over time.

Peripheral lymphocyte preparation

Buffy coats containing peripheral blood lymphocytes (PL) were extracted as previously described by our laboratory.[21] Cell density was adjusted to 1×10^6 cells/ml with the trypan blue exclusion test.

DNA extraction

Genomic DNA was extracted from PLs for each patient. Cells were transferred to 500 µl lysis buffer containing 0.5% SDS, 150 mM NaCl, 10 mM EDTA, and 10 mM Tris-HCl (pH 8.0). To this, RNase A (100 µg/ml, DNase-free) was added, and the solution was incubated at 37°C for 1 hour. Following the RNase A step, proteinase K (200 µg/ml) was added to the solution and thereafter incubated for 3 hours at 50°C. Protein contaminants were then precipitated by addition of 0.1 volume 5 mM potassium acetate and centrifuging (5 000 ×g, 15 minutes). Supernatants containing genomic DNA were transferred to fresh tubes and extracted with 100% isopropanol on ice and then washed with 70% ethanol. DNA samples were then dissolved in 10 mM Tris and 0.1 mM EDTA (pH 7.4) at 4°C overnight. To verify DNA extraction, equal amounts of DNA (300 ng) were electrophoresed (150 V, 50 min.) on a 1.8% agarose gel containing 0.5 mg/ml ethidium bromide. DNA bands were visualised by UV light and digitally photographed using a gel documentation system (Chemi-Doc XRS, Bio-Rad) and Quantity One Image Analysis software (Bio-Rad). The concentration of each sample was determined spectrophotometrically.

Genotyping for the -308 TNF-α promoter polymorphism

Polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) was used to determine the -308 TNF-α promoter polymorphism. A 107bp PCR product was amplified using 20 pmol of forward and reverse primer in a 25 µl reaction containing 0.2 mM of each dNTP, 1.5 mM $MgCl_2$, 1X Green GoTa0071 Flexi buffer (Promega), 1 U GoTaq DNA polymerase (Promega) and 100 ng genomic DNA template. The forward and reverse primers were those according to Wilson *et al.*[20] (5'AGGCAATAGGTTTTGAGGGCCAT 3'; 5' TCCTCCCTGCTCCGATTCCG 3').

DNA was amplified for 35 cycles with denaturation at 94°C for 3 minutes, annealing at 60°C for 1 minute, extension at 72°C for 1 minute and a final extension at 72°C for 5 minutes. The PCR product was then digested with the restriction enzyme *Nco*I for 12 hours at 37°C. Digestion of the PCR product confirmed 2 alleles viz. -308 G allele which resulted in 2 fragments (87 bp and 20 bp), and –308 A allele which resulted in a single 107 bp fragment (Fig. 1).[20]

TNF-α enzyme-linked immunosorbent assay (ELISA)

Plasma was collected by centrifuging whole blood. Plasma TNF-α concentration was measured using the human TNF-α Max

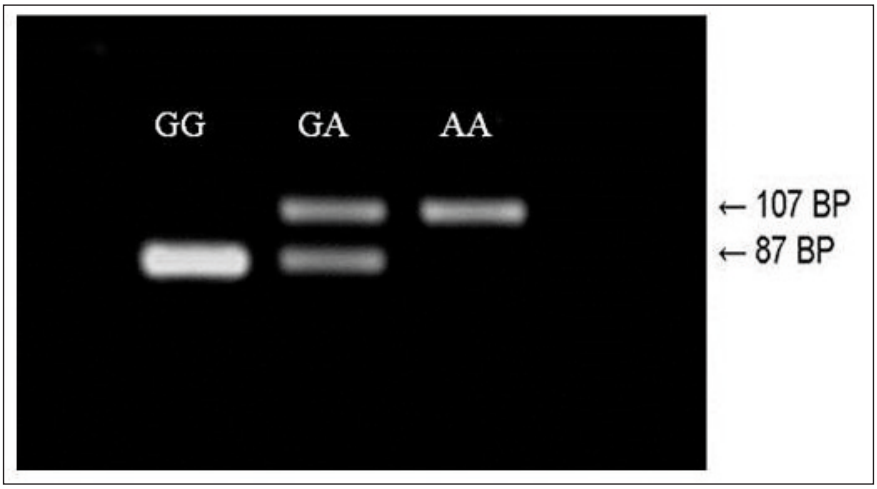

Fig. 1. Restriction fragment length polymorphism (RFLP) showing alleles of the -308 TNF-α promoter polymorphism. The -308 G allele gave rise to a 87 bp and 20 bp fragment, and the -308 A allele to a 107 bp fragment.

Standard ELISA kit (Biolegend). A high-affinity microtitre plate was coated with TNF-α capture antibody (100 µl/well, 18 hours at 4°C). Plates were washed and treated with 200 µl assay diluent. Thereafter, 100 µl standards and samples were added. Biotinylated anti-human TNF-α detection antibody and avidin-horseradish peroxidase were then added, followed by the TMB substrate and the stop solution. Absorbance was measured at 450 nm (570 nm reference) (Bio-Tek µQuant ELISA plate reader). Plasma concentrations of TNF-α were calculated by extrapolation from the standard curve.

CD4 T_H cell apoptosis, CD4 T_H cell counts and viral loads

CD4 T_H lymphocyte apoptosis, CD4 T_H cell counts and viral loads were determined as described previously.[21]

Statistical analysis

Genotype and allelic frequencies of the TNF-α -308 polymorphism for the control and HIV-1 cohort were compared by direct counting. Hardy-Weinberg statistics were used to determine whether our study cohort was representative of the larger population. Statistical analyses and correlations were done using Graphpad Prism Software (version 5).

Results

-308 TNF-α promoter polymorphism

Genotypic distribution did not deviate from those predicted by the Hardy-Weinberg equilibrium (HIV-1: p=0.331, chi-square statistic=0.946; controls: p=0.194, chi-square statistic=1.688). There were no significant differences in genotypic distribution between the HIV-1 and control cohorts respectively (GG 60% and 65.8%; GA 37.3% and 27.6%; and AA 2.7% and 6.6%). However, when allelic distribution was investigated, we found that the -308 G allele was more frequent in the control population (79.6% v. 78.7%) but this difference did not reach statistical significance (chi square test p=0.888, odds ratio=1.06, 95% CI (confidence interval) 0.607 - 1.84; see Table 1).

Table 1. Genotypic and allelic frequencies of the -308 TNF-α promoter region polymorphism in both HIV-positive and control populations

	HIV (N=75)	p value	Controls (N=76)	p value
Genotype frequency				
G/G	60%	0.331*	65.8%	0.194*
G/A	37.3%		27.6%	
A/A	2.7%		6.6%	
Allelic frequency				
A	21.3%		20.4%	0.888
G	78.7%		79.6%	

*Hardy-Weinberg equilibrium (HIV-1: chi-square statistic=0.946; controls: chi-square statistic=1.688).

Table 2. Mean TNF-α concentration and markers of HIV-1 progression in the HIV-positive and control cohorts. Markers of HIV-1 progression within the -308 GG and -308 GA genotypes of the HIV-positive cohort

	HIV-positive patients	Controls	p value
TNF-α concentration (pg/ml)	10.87±0.73 (14.40)	3.57±1.36 (0.00)	p<0.0001
% apoptosis of $CD4^+$ T cells	25.98±1.82 (24.30)	8.52±0.90 (6.94)	p<0.0001
HIV-positive patients	GG	GA	
TNF-α concentration (pg/ml)	15.01±1.40 (14.04)	15.52±1.05 (15.39)	p=0.403
Plasma viral load (log copies/ml)	3.69±0.337 (4.66)	3.92±0.321 (4.36)	p=0.970
$CD4^+$ T cell count (cells/µl)	256.10±25.04 (243.00)	288.60±20.97 (275.00)	p=0.242
% apoptosis of $CD4^+$ T cells	28.04±2.57 (24.52)	22.57±2.45 (23.30)	p=0.223

All values reported as mean±SEM (median).

Plasma TNF-α concentration

Mean plasma TNF-α concentration was determined in patients and controls by ELISA. The HIV-1 infected subjects showed significantly higher TNF-α concentration than controls (10.87 pg/ml and 3.57 pg/ml, p<0.0001, 95% CI: HIV-1 infected patients 9.39 - 12.36 pg/ml, controls 0.74 - 6.41 pg/ml; see Table 2).

We then investigated whether genotypic variation at the -308 locus influenced plasma TNF-α concentration in the HIV-1 infected cohort. Mean TNF-α concentrations were determined after grouping patients according to genotype. Higher plasma TNF-α concentrations were recorded in the -308GA genotype than in the -308GG genotype (15.52 pg/ml v. 15.01 pg/ml). This difference did not reach statistical significance (Mann-Whitney test, p=0.404, 95% CI: GA 13.35 - 17.70 pg/ml, GG 12.19 - 17.83 pg/ml; see Table 2). The mean TNF-α concentration in patients with the -308AA genotype was 19.35 pg/ml.

Genotype and clinical parameters

Since genotypic differences in TNF-α concentration were noted, we investigated whether genotype influenced viral load and CD4 T_H cell counts. Lower mean plasma viral load and lower mean CD4 T_H cell counts were observed in the -308GG genotype than in the -308GA genotype (3.69 log copies/ml v. 3.92 log copies/ml and 256.10 cells/µl v. 288.60 cells/µl respectively), with no significant difference (Mann-Whitney, p=0.970, 95% CI: GG 3.00 - 4.38 log copies/ml, GA 3.25 - 4.58 log copies/ml and p=0.242, 95% CI: GG 204.80 - 307.40 cells/µl, GA 245.30 - 331.90 cells/µl; Table 2). Mean plasma viral load and CD4 T_H cell counts in patients with the -308AA genotype were 3.59 log copies/ml and 197.00 cells/µl respectively.

Genotype and HAART

Following the observation of genotypic differences in the clinical markers of infection, we investigated whether genotype influenced patient response to treatment. Patients were grouped into HAART-naive and HAART-treated cohorts, and these groups further

stratified according to genotype. Mean plasma viral load and CD4 T_H cell counts were analysed according to genotype and treatment.

In the HAART-naive cohort, higher plasma viral loads and lower CD4 T_H cell counts were observed in the -308GG genotype than in the -308GA genotype (4.92 log copies/ml v. 4.54 log copies/ml and 244.30 cells/µl v. 283.80 cells/µl) but there were no significant differences (Mann-Whitney test, p=0.101, 95% CI: GG 4.68 - 5.16 log copies/ml, GA 4.17 - 4.90 log copies/ml and p=0.250, 95% CI: GG 179.70 - 308.80 cells/µl, GA 233.80 - 333.80 cells/µl; see Table 4).

Higher CD4 T_H cell counts and statistically significant lower plasma viral loads were recorded in the HAART-treated cohort than in the HAART-naive cohort (288.64 cells/µl v. 264.80 cells/µl and 1.19 log copies/ml v. 4.72 log copies/ml) (Mann-Whitney test, p=0.451, 95% CI: HAART-naive 226.80 - 302.80 cells/µl, HAART-treated 216.70 - 360.60 cells/µl and p<0.0001, 95% CI: HAART-naive 4.51 - 4.93 log copies/ml, HAART-treated 0.940 - 1.44 log copies/ml; Table 3). This result was expected as HAART is associated with lower plasma viral loads and higher CD4 T_H cell counts. Interestingly, we noticed genotypic differences in the HAART-treated cohort in the -308GG genotype. The -308GG genotype showed higher plasma viral loads and lower CD4 T_H cell counts than in the -308GA genotype (1.22 log copies/ml v. 1.13 log copies/ml and 278 cells/µl v. 314.0 cells/µl); however, the differences did not reach statistical significance (Mann-Whitney, p=0.251, 95% CI: GG 0.855 - 1.58 log copies/ml, GA 1.02 - 1.23 log copies/ml and p=0.374, 95% CI: GG 177.70 - 379.30 cells/µl, GA 185.40 - 442.60 cells/µl; see Table 4).

Genotype and apoptosis

Since genotypic differences were observed in TNF-α concentration, we investigated whether genotype influenced CD4 T_H cell apoptosis. Significantly higher mean apoptosis levels were observed in HIV-1 infected patients than in controls (25.98% v. 8.52%; Mann-Whitney test, p<0.0001, 95% CI: control 6.71 - 10.32%, HIV-1 infected 22.35 - 29.61%; see Table 2). In the HIV-1 cohort, higher apoptosis levels were observed in the -308GG genotype (28.04%); however, there was no statistical difference between genotypes (Mann-Whitney, p=0.223, 95% CI: GG 22.87 - 33.21%, GA 17.56 - 27.58%; see Table 2).

We investigated mean apoptosis levels in patients on treatment, and observed higher apoptosis levels in the HAART-naive cohort than in the HAART-treated HIV-1 infected cohorts; however, the differences did not reach statistical significance (27.13% v. 23.68%, Mann-Whitney test, p=0.482, 95% CI: HAART-naive 22.14 - 32.13%, HAART treated 18.99 - 28.38%; see Table 3). The -308GG genotype showed higher apoptosis levels in both the HAART-naive and HAART-treated HIV-1 infected cohorts than in the -308GA genotype (32.12% v. 29.58% and 23.77% v. 21.57%); however, differences in both cohorts were not statistically significant (Mann-Whitney test, p=0.404, 95% CI: GG 25.17 -39.07%, GA 22.79 - 36.37% and p =0.786, 95% CI: GG 18.19 - 29.35%, GA 4.82 - 38.32%; see Table 4). The mean apoptosis level in the patients with the -308AA genotype was 27.77%.

Table 3. Markers of HIV progression in HAART-naive and HAART-treated groups in HIV-positive patients

	HAART-naive	HAART-treated	*p* value
Plasma viral load (log copies/ml)	4.72±0.105 (4.85)	1.19±0.115 (1.06)	p<0.0001
CD4⁺ T cell count (cells/µl)	264.80±18.80 (256.00)	288.64±33.31 (293.00)	p=0.451
% apoptosis of CD4⁺ T cells	27.13±2.49 (24.77)	23.68±2.27 (22.86)	p=0.482

All values reported as mean±SEM (median).

Discussion

TNF-α is an immune regulatory cytokine that is released in response to viral antigens to combat infection.[10-12] However, chronically high concentrations of TNF-α may facilitate progression of HIV-1 and apoptosis of bystander T cells.[22]

Table 4. Markers of HIV progression in the -308 GG and -308 GA genotypes in the HAART-naive and HAART-treated groups

	GG	GA	*p* value
HAART-naive			
Plasma viral load (log copies/ml)	4.92±0.115 (4.91)	4.54±0.173 (4.57)	p=0.101
CD4⁺ T cell count (cells/µl)	244.30±30.72 (188.00)	283.80±23.97 (273.00)	p=0.250
% apoptosis of CD4⁺ T cells	32.12±3.40 (26.49)	29.58±3.34 (24.54)	p=0.404
HAART-treated			
Plasma viral load (log copies/ml)	1.22±0.16 (1.06)	1.13±0.03 (1.15)	p=0.251
CD4⁺ T cell count (cells/µl)	278.50±44.57 (273.50)	314.00±40.42 (314.00)	p=0.374
% apoptosis of CD4⁺ T cells	23.77±2.67 (22.36)	21.57±5.26 (22.48)	p=0.786

All values reported as mean±SEM (median).

TNF-α indirectly induces viral replication by activating NF-κB[23] which binds to the long terminal repeat (LTR) of HIV.[23,24] This may lead to production of viral proteins such as Tat and Nef which further induce TNF-α production via the inflammatory response.[23,24] The -308 TNF-α promoter polymorphism has been associated with altered TNF-α concentration.[15,16] Genotypic variation may induce conformational changes in the promoter region that increase binding affinity of transcription factors, such as NF-κB.[15,16,23]

Ours is the first report on the -308 TNF-α promoter polymorphism in HIV-1 infected black South Africans. It is probable that elevated levels of TNF-α may alter clinical outcomes in the patient.[4,17] A previous study showed lower CD4 T_H cell apoptosis and plasma viral load in a cohort of HIV-1 infected patients on HAART.[21] The current study aimed to investigate whether the -308 TNF-α promoter polymorphism influenced TNF-α concentration, CD4 T_H cell count, CD4 T_H cell apoptosis and plasma viral load in HIV-1 infected black South Africans.

It is well established that TNF-α concentration is elevated early in infection.[10,11] However, during HIV-1 infection, consistently high levels of TNF-α may be attributed to constant antigenic stimulation from viral proteins such as Tat and Nef.[23,24]

Our study shows that the -308 G allele was similar in both the HIV-1 infected and control cohorts. This finding is consistent with other studies that reported similar allelic frequencies in different demographic groups.[4,25-27] The -308 G allele in the HIV-1 infected cohort was associated with significantly high levels of TNF-α, which may be due to increased binding affinity of transcription factors.

In addition to high TNF-α concentration, this study showed a cross-sectional association between allelic frequency and markers of HIV disease progression, which was indicated by high bystander T_H cell apoptosis and viral replication. High TNF-α concentration is involved in HIV-1 replication via clonal expansion of infected T_H cells.[13,23] It is also involved in rapid apoptosis of bystander T_H cells, which may account for the high viral titres and high levels of apoptosis observed in this study. During HIV-1 infection, TNF-α may act as a molecular rheostat that switches between clonal expansion and bystander T_H cell apoptosis, depending on membrane receptor profile.[1,14] Genotypic differences in the TNF-α promoter that influence a cell's inherent ability to produce the cytokine may exacerbate these functions during HIV-1 infection. In response to rapid apoptosis, the immune system may compensate by increasing bone marrow turnover of mononuclear cells. These may, however, not reach complete maturation and lead to impaired T_H cell recovery, ultimately contributing to HIV-1 progression.[1,22,28]

This study differs from previous studies which have associated the -308 A allele with high TNF-α concentration and disease.[4,17,19] The -308AA genotype has been widely associated with poorer clinical outcomes and disease progression in *Leishmaniasis*, cerebral malaria and insulin-dependent diabetes mellitus.[29-31] Interestingly, some reports showed no association between this genotype and disease severity.[25-27,32,33] In studies that showed the association between the -308AA genotype and disease severity, frequencies of the -308 A allele were low, which may have conferred low statistical power and, as such, these conclusions warrant confirmation in other populations.[4,34] Furthermore, the bulk of these studies were performed in populations of white ancestry. No studies to date have investigated the influence of the -308 TNF-α promoter polymorphism in infectious diseases in a black African population.

Conclusion

In contrast with other studies, our study reports for the first time that the -308 G allele may contribute to mechanisms that lead to poorer response to HAART therapy in Black South Africans infected with HIV-1. Similarly, we found the -308AA genotype to be least frequent (*N*=2), which may preclude disease association studies until adequate sample sizes are collected. Comparable clinical outcomes were observed in heterozygote individuals, providing further evidence that the presence of the -308 G allele may be associated with markers of HIV-1 progression in this study.

Single nucleotide polymorphisms that affect regulation of cytokines may affect host response to HIV-1 infection. This effect may influence disease progression and clinical outcomes. To provide holistic management of patients infected with HIV-1 and develop individual treatment strategies, it is imperative to study genotypic differences between individuals. Such approaches may curb the advent of adverse drug reactions, minimise therapeutic failures and also address not only the medical, but also the economic burdens of this disease.

Acknowledgements. The authors thank LIFElab for funding.

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