Scarring Trachoma Is Associated with Polymorphism in the Tumor Necrosis Factor Alpha (TNF-α) Gene Promoter and with Elevated TNF-α Levels in Tear Fluid

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Tumor necrosis factor alpha (TNF-α) may play a central role in the disease pathogenesis which occurs as a consequence of chlamydial infection. To investigate the importance of *TNF-α* gene promoter polymorphisms and TNF-α levels in tear fluid in scarring trachoma, a large matched-pair case-control study was performed in The Gambia. The *-308A* allele was present in a higher proportion of patients (28.4%) than controls (18.4%), with an increasing association for homozygotes (χ^2 for trend, $P = 0.032$; allele frequency, 0.163 in patients and 0.099 in controls; χ^2, $P = 0.025$). For the *-238A* allele, the association was similar but not significant. The disease association was highly significant when the number of either *-308A* or *-238A* sites in an individual was considered ($P = 0.003$). *TNF-α* promoter alleles are tightly linked to some HLA class I and II alleles, but multivariate analysis confirmed that the disease associations were independent of HLA, although a class I allele, *A*6802*, is also associated with disease. TNF-α was more frequently detected in tear samples from patients (27.6%) than from controls (15.9%), increasingly so for higher levels of detectable TNF-α ($P = 0.015$). Among patients, detectable TNF-α in tears was highly associated with the presence of ocular chlamydial infection ($P < 0.001$). The results indicate that TNF-α plays a major role in the tissue damage and scarring which occurs as a consequence of *Chlamydia trachomatis* infection.

Tumor necrosis factor alpha (TNF-α) may play a central role in the pathogenesis of chlamydial disease. Persistent or repeated infection with the intracellular bacterium *Chlamydia trachomatis* leads to pathogenic changes at the ocular or genital site of infection (10, 11, 18, 23). Chronic inflammatory responses to the epithelial infection cause tissue damage (31), leading to fibrosis and scar formation. Scarring of the upper eyelid causes inversion and trichiasis, which permanently damages the cornea and may lead to blindness (18, 23), whereas scarring of the fallopian tube causes tubal occlusion and consequent infertility or ectopic pregnancy (10, 11).

Murine *C. trachomatis* infection primes spleen cells for in vitro production of TNF-α, which is cytotoxic to cultured fibroblasts (34) and thus may contribute directly to tissue damage in vivo. TNF-α is an important mediator of inflammation and may be induced by the lipopolysaccharide endotoxin of *C. trachomatis* (21). Reverse transcription-PCR has been used to detect TNF-α mRNA in conjunctival swabs from patients with trachomatous inflammation (4). TNF-α is also known to be involved in processes of fibrogenesis and tissue remodelling, including stimulation of prostaglandin E_2, glycosaminoglycan, and collagenase production by fibroblasts (15, 17, 33), and plays a central role in experimental pulmonary fibrosis induced by bleomycin (25) or silica (26). Thus, it is reasonable to suggest that TNF-α could be important in the fibrogenic process of scarring in chlamydial disease.

Conversely, TNF-α may protect against active chlamydial infection, as it has an antichlamydial effect both in vitro (29, 30) and in vivo against the *C. trachomatis* mouse pneumonitis agent in the lung (35). Secretion of TNF-α occurs at the genital site of *C. psittaci* infection in guinea pigs (14), although its relevance to protection is unknown.

TNF-α production may be regulated at the transcriptional, posttranscriptional, and translational levels (16, 19, 28). Polymorphisms in the TNF-α gene may affect transcriptional regulation, because levels of secretion of TNF-α by human monocytes and peripheral blood mononuclear cells (PBMCs) in vitro are associated with extended HLA haplotypes and microsatellite alleles linked to *TNF-α* in the major histocompatibility complex class III region (1, 27). Single-base polymorphisms have been identified at positions -308 and -238 in the human *TNF-α* gene promoter (13, 36), with the rarer allele containing A instead of G at each site. The -238 polymorphism is in a putative transcriptional regulation Y-box sequence (13), and in vitro experiments are being used to test whether the -308 polymorphism affects transcription (7, 37). These polymorphisms are associated, independently of linked HLA polymorphisms, with diseases in which TNF-α is involved in pathogenesis: the homozygous *TNFA-308A* genotype is associated with cerebral malaria (24), and there is a higher than expected *TNFA-308A* allele frequency among patients with mucocutaneous leishmaniasis (8).

Understanding the processes involved in the pathogenesis of chlamydial disease is important, to help design future immunoprophylactic or therapeutic approaches to disease control. To investigate whether polymorphism in the *TNF-α* gene promoter and levels of TNF-α in tear fluid are involved in pathogenesis of scarring trachoma, a large case-control study was performed.

MATERIALS AND METHODS

Case-control study subjects and sample collection. One hundred fifty-three patients with scarring trachoma were identified by clinical examination. These

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subjects were recruited from among those who had been referred for eyelid surgery at Kaur Health Centre, McCarthy Island Division, The Gambia, from other inhabitants of their villages, and from inhabitants of two trachoma study villages, Jali and Berending. Their mean age was 34 years (range, 6 to 70 years). One hundred six (69%) were female. Ethnically, 111 (73%) were Wolof, 26 (17%) were Mandinka, 10 (7%) were Fula, and 6 (4%) were Jola. For each case, a control subject with normal eyelids who was matched for age ($<$5-year difference), sex, and village of residence was recruited. Most of the villages were inhabited by a single ethnic group, and each control was from a different family from that of the patient. From each subject, a 10-ml sample of venous blood was collected into heparin, and PBMCs were separated with Ficoll-Hypaque. A proportion of the PBMCs were used for HLA class I serological typing (12), and the remainder were kept for extraction of DNA. A sample of tear fluid was obtained from each subject by placing a piece of cellulose sponge onto the lower eyelid for approximately 30 s, a method which typically absorbed 50 to 100 μl of tear fluid. A swab was collected from the subtarsal conjunctiva of the right eye of each subject and stored in 1 ml of transport medium. PBMC, tear, and eye swab samples were frozen at $-20°C$ within 3 h of collection and subsequently transported on dry ice and stored at $-70°C$.

TNF-α promoter genotyping. DNA was extracted from the PBMC samples of all subjects. The *TNFA-308* G/A polymorphism was typed by PCR amplification followed by sequence-specific oligonucleotide (SSO) probing, as described by McGuire et al. (24). One hundred thirty of the subjects were also typed by the PCR-restriction fragment length polymorphism (RFLP) method of Wilson et al. (36) to confirm the accuracy of the PCR-SSO genotyping. The *TNFA-238* G/A polymorphism was typed by PCR-SSO with the same PCR products as for the *TNFA-308* typing and SSO probes described by D'Alfonso and Richiardi (13).

TNF-α detection in tear fluid. The cellulose sponge tear sample from each subject was eluted with 600 μl of buffer (Tris buffer [pH 7.5], 1% bovine serum albumin, and the following protease inhibitors: 1 mM EDTA, 1 mM EGTA, 1 mM N-ethylmaleimide, 1 mM phenylmethylsulfonyl fluoride, 0.1 mM N-tosyl-amide-L-phenylalanine chloromethyl ketone, and 1 μm pepstatin A). This eluate was thus very approximately a 10-fold dilution of the tear fluid collected. A highly sensitive assay of the TNF-α concentration in the eluate was performed with the QUANTIKINE HS enzyme-linked immunosorbent assay (ELISA) kit (R&D Systems). The samples were tested in duplicate, and the concentrations were calculated from optical density values by using a standard curve of doubling dilutions of recombinant TNF-α on each plate from 0.5 to 16 pg ml^{-1}.

PCR detection of *Chlamydia* infection. PCR amplification was performed to detect the presence of chlamydial plasmid DNA in eye swab samples (2). This is a sensitive diagnostic method for the presence of ocular infection with *Chlamydia*.

Statistical methods. Tests on proportions between groups were performed by the χ^2 test or Fisher's exact test, with EPIINFO version EPI-6 software. To test for directional, dose-dependent associations, χ^2 for trend was performed (22). Univariate and multivariate analyses to test for disease associations, taking into account the matching of case-control pairs, were performed by conditional logistic regression (CLR), with EGRET software. The magnitudes of associations were estimated as odds ratios with 95% confidence intervals (OR, 95% CI).

RESULTS

TNF-α promoter polymorphisms and scarring trachoma. The G/A polymorphisms at positions -308 and -238 of the TNF-α gene promoter were determined. The *TNFA-308A* allele frequency was higher among trachoma patients than controls (0.163 and 0.099, respectively; $P = 0.025$). The *TNFA-238A* allele frequency was also higher among patients, although not significantly (0.091 and 0.052, respectively; $P = 0.075$). Table 1 shows that a higher proportion of patients (28.4%) than controls (18.4%) had the *TNFA-308A* allele, particularly as homozygotes (χ^2 for trend = 4.57; $P = 0.032$). A similar but nonsignificant trend was seen for the less common *TNFA-238A* allele. The trend for the number of either *TNFA-308A* or *TNFA-238A* sites was highly significant (χ^2 for trend = 8.64; $P = 0.003$). Although they are closely situated within the TNF-α promoter, the *TNFA-308A* and *TNFA-238A* polymorphisms were not associated with each other among individuals (OR and 95% CI = 0.41 and 0.12 to 1.14, respectively).

The *TNFA-308A* and *TNFA-238A* alleles were significantly associated with particular HLA class I and class II types (Table 2). One HLA type had previously been shown to be associated with scarring trachoma in these case-control subjects, i.e., HLA-A28 (due to the allele subtype *HLA-A*6802*) (12), and this was negatively associated with the *TNFA-308A* allele (OR

and 95% CI = 0.26 and 0.08 to 0.69, respectively; $P = 0.003$). The subtype *HLA-A*6802* was similarly negatively associated with *TNFA-308A* but at a lower level of significance (OR and 95% CI = 0.26 and 0.05 to 0.89, respectively; $P = 0.021$). As might be expected from the negative association between the alleles, the disease association of *TNFA-308A* was independent of *HLA-A*6802* as determined by multivariate CLR analysis (Table 3).

TNF-α in tear fluid of patients with scarring trachoma and controls. TNF-α was more frequently detected (>0.5 pg ml^{-1}) in eluates of tear samples from patients (27.6%) than from controls (15.9%) (CLR: OR and 95% CI = 2.5 and 1.20 to 5.20, respectively; $P = 0.013$). This association was stronger for higher levels of detectable TNF-α (Table 4). The presence of detectable TNF-α in tear samples was not associated with either of the TNF-α gene promoter polymorphisms (for *TNFA-308A* and *TNFA-238A*, respectively, OR = 0.51 and 0.72, $P = 0.12$ and 0.62), and its association with disease was not affected by incorporation of the promoter genotypes in a multivariate

TABLE 1. Proportions of trachoma patients and controls with different *TNF-α* promoter genotypes (positions -308 and -238)

Genotype and no. of matched pairs	% of:		χ^2 for trend, P	CLR[a]	
	Patients	Controls		OR	95% CI
TNFA-308 genotype ($n = 141$)					
-308 G/G	71.6	81.6	0.032	1.0	
-308 A/G	24.1	17.0		1.59	0.87–2.90
-308 A/A	4.2	1.4		3.40	0.67–17.1
TNFA-238 genotype ($n = 143$)					
-238 G/G	83.9	90.2	0.09	1.0	
-238 A/G	14.1	9.1		1.62	0.75–3.48
-238 A/A	2.1	0.7		3.42	0.35–33.6
No. of *-308A* or *-238A* sites ($n = 136$)					
0	58.8	74.3	0.003	1.0	
1	32.4	22.8		1.56	0.90–2.70
2	8.8	2.9		3.28	1.05–10.3
>2	0	0			

[a] CLR analysis of matched case-control pairs.

TABLE 2. HLA types associated with the *TNFA-308A* and *TNFA-238A* alleles among study subjects[a]

TNF allele	HLA type	CLR		P
		OR	95% CI	
TNFA-308A	A28	0.26	0.08–0.69	0.003
	B70	3.70	1.85–7.44	0.00004
	Cw2	3.02	1.56–5.83	0.0003
	DRβ1*11	2.24	1.20–4.18	0.006
	DRβ1*1303	0.0	0.0–0.66	0.009
TNFA-238A	B53	7.14	3.19–16.21	<0.00000001
	Cw5	7.36	2.36–22.94	0.0004
	Cw6	6.95	2.81–17.21	0.00001
	DRβ1*09	9.22	3.77–22.72	0.0000004

[a] Associations for which $P < 0.01$ are shown. Tests were performed only for HLA types with an overall prevalence of $>5\%$ (i.e., nine HLA-A types, eight HLA-B types, seven HLA-Cw types, eight DRβ1 types, and six DQβ1 types). HLA typing of the case-control subjects was performed previously (12).

TABLE 3. Joint effects of alleles at two sites in the *TNF-α* promoter and a class I HLA allele on scarring trachoma case-control status

Presence of allele	Multivariate CLR model[a]		*P*
	OR	95% CI	
TNFA-308A	2.09	1.11–3.95	0.023
TNFA-238A	1.79	0.82–3.91	0.142
HLA-A*6802	3.86	1.57–9.49	0.003

[a] CLR analysis of 130 matched case-control pairs typed for the three loci.

CLR analysis (adjusted OR and 95% CI = 2.4 and 1.2 to 5.0, respectively; $P = 0.017$).

Presence of chlamydial infection determined by PCR. A total of 12 of 150 patients (8.0%) and 0 of 153 controls had PCR-detected chlamydial plasmid DNA in eye swabs (Fisher's exact test, $P < 0.001$). A total of 9 (75%) of the 12 patients with current chlamydial infection had detectable TNF-α in tear fluid, compared with 29 (22.1%) of 131 patients without chlamydial infection (Fisher's exact test, $P < 0.001$). None of the 12 patients with current chlamydial infection had the *TNFA-308A* allele, and 2 of 11 tested had the *TNFA-238A* allele (Fisher's exact test, $P = 0.07$ and 1.0, respectively [not significant]).

DISCUSSION

The results of this study suggest that TNF-α plays a major role in the pathogenesis of scarring trachoma. The *TNF-α* gene promoter *TNFA-308A* allele was significantly associated with disease, with an apparently higher risk for homozygotes. The rarer *TNFA-238A* allele showed a similar trend, which was not significant. The number of either *TNFA-308A* or *TNFA-238A* sites in an individual was highly significantly associated with disease. The disease association with the *TNFA-308A* allele was not due to linkage with HLA class I or II alleles and was independent of a disease association with the class I allele *A*6802* (12).

TNF-α was more frequently detected in tear fluid samples from patients with scarring trachoma than from controls. Among patients, TNF-α was detected much more frequently in samples from those with evidence of current ocular chlamydial infection, indicating that TNF-α levels are particularly high during infection. The observed low prevalence of active ocular chlamydial infection among patients with scarring trachoma was expected, because most of the subjects were adults and infection is normally more common among children (32). However, the prevalence of infection among patients with scarring trachoma was higher than among controls, in whom no infections were detected, consistent with a hypothesis that the

TABLE 4. Proportions of trachoma patients and controls with detectable levels of TNF-α in eluted tear samples[a]

Amt of TNF-α (pg/ml)	% of:		χ² for trend, *P*	CLR[b]	
	Patients (*n* = 145)	Controls (*n* = 145)		OR	95% CI
<0.49	72.4	84.1	0.015	1.0	
0.5–0.99	15.2	9.7		2.10	1.0–4.44
1.0–4.99	9.6	4.8		3.15	1.10–9.06
>5.0	2.8	1.4		3.91	0.63–24.1

[a] Tear fluid was collected on cellulose sponges and eluted into buffer, yielding an approximately 10-fold dilution (as described in Materials and Methods).
[b] CLR analysis of 145 matched case-control pairs.

patients are less able to resolve *C. trachomatis* infections (3, 20). Almost all patients (90%) and controls (86%) had evidence of exposure, indicated by serum antibodies to *C. trachomatis* measured by microimmunofluorescence (12).

Detection of TNF-α in tear fluid samples was not associated with particular *TNF-α* promoter genotypes. If the promoter genotypes are involved in differential transcriptional regulation, it does not necessarily follow that transcription is induced more readily or at higher levels for one allele than another. Transcriptional regulation of TNF-α is probably under complex homeostatic control, as is the case for the TNF-α receptor (16). One allele may be less sensitive to homeostatic down-regulation and thus may allow transcription to proceed for longer causing more persistent production of TNF-α. Further in vitro studies are required to examine the effects of the different promoter alleles on transcription (7, 37). It is also apparent that some control of TNF-α production is at the posttranscriptional (28) and translational (19) levels. Subjects were sampled only once in this study, and it is probable that measurement of mean TNF-α levels in multiple samples over time would give a more precise estimation of TNF-α secretion in each subject.

Studies on chlamydial infection in animal models indicate that TNF-α levels reflect the intensity of current infection (14) and may cause tissue damage which leads to scarring (34). The likely additional role of TNF-α in the fibrogenic process of scar formation (25, 33) has not yet been investigated in experimental models of chlamydial disease. Transforming growth factor β (TGF-β) is a key cytokine controlling fibrogenesis (5), and it may be important to understand regulatory interactions between TNF-α and TGF-β. Conjunctival swabs from Tanzanian patients with trachomatous scarring contain elevated levels of TGF-β mRNA compared to swabs from controls without trachoma (4).

The possibility that TNF-α also contributes to the resolution of active chlamydial infection is not excluded. The antichlamydial effect previously observed in vitro (29, 30) and in vivo (35) was modest compared with that exhibited by gamma interferon (IFN-γ). However, the antichlamydial activity of TNF-α was synergistic with IFN-γ (29, 30), and so it might contribute to clearance of infection. A small number of eluted tear samples in the present study were assayed for IFN-γ by a moderately sensitive ELISA, but levels were undetectable. A longitudinal study of acquisition and resolution of chlamydial infection in children and a more sensitive assay for IFN-γ could address whether tear fluid IFN-γ and/or TNF-α is involved in clearance of infection.

The *TNFA-308A* allele, associated with scarring trachoma in this study, has also been associated with pathogenesis (independently of HLA polymorphisms) in other infectious diseases (8, 24). Given these disease associations, it is unclear why this allele is allowed to exist at a fairly high frequency in human populations. It is possible that in some situations the heterozygous genotype is advantageous or that being homozygous for the common allele *TNFA-308G* is associated with a risk of other conditions (6, 9). It is also possible that tightly linked and yet unidentified alleles at other loci modify the production or activity of TNF-α and influence the frequency of particular major histocompatibility complex class III region haplotypes in particular disease groups.

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