

# Susceptibility to sequelae of human ocular chlamydial infection associated with allelic variation in *IL10* cis-regulation

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**Trachoma, an infectious disease of the conjunctiva caused by *Chlamydia trachomatis*, causes scarring and blindness in some infected individuals but not others. In an African community where trachoma is endemic, we have previously identified an IL10 haplotype that is associated with increased risk of scarring complications. Here we examine the hypothesis that the risk haplotype (H-RISK) affects levels of IL10 expression in the conjunctiva during active trachoma infection. To overcome potential genetic and environmental confounders we used the method of allele-specific quantification, which involved identifying subjects in the community who had active trachoma and were also heterozygous for the H-RISK. We find that there is allelic variation in cis-regulation of IL10 in the conjunctiva during active trachoma, with the H-RISK generating relatively more IL10 transcripts than other haplotypes in this population (average difference in IL10 allelic transcripts in the conjunctiva of heterozygous individuals infected with *C. trachomatis* of 23% (95% confidence interval: 14–32%,  $P < 0.0001$ ). These findings provide a plausible functional explanation for the observed genetic association, and support the hypothesis that an excessive IL10 response to *C. trachomatis* infection is a risk factor for scarring and blindness.**

## INTRODUCTION

Trachoma is an infectious disease of the conjunctiva caused by *Chlamydia trachomatis*, and an important cause of blindness, particularly in developing countries. The World Health Organization (WHO) estimates that 100 million people are affected by trachoma and seven million are blind or visually impaired as a result (1,2). Blindness and visual impairment result from fibrosis and scarring due to the repair of tissue damaged by chlamydia-induced inflammation. It is not understood why this inflammatory process develops in some infected individuals and not others.

*C. trachomatis* is also the commonest bacterial sexually transmitted infection (3). In some but not all infected women (~10–30%) it progresses to pelvic inflammatory disease, infertility or ectopic pregnancy if untreated. For both

ocular and genital *C. trachomatis* infection, the factors involved in exacerbated inflammatory disease and pathological scarring are not well understood.

In trachoma, *C. trachomatis* infection and scarring occur in the conjunctiva, which is readily accessible to examination and investigation. After the initial infection, the disease may resolve or progress through active trachomatous inflammation to a chronic inflammatory process that leads to scarring after repeated or persistent episodes of infection (4). Severe scarring causes the lashes to turn inwards and abrade the cornea (trichiasis), which leads eventually to blindness (2). Even in highly trachoma endemic communities only a minority of individuals develop severe scarring and trichiasis.

By means of case-control studies, we have identified a number of genetic polymorphisms associated with increased risk of scarring sequelae (5–10). One such association is

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**Table 1.** Summary of genotyped haplotype tagging SNPs in the 10 kb genomic region across the *IL10* gene

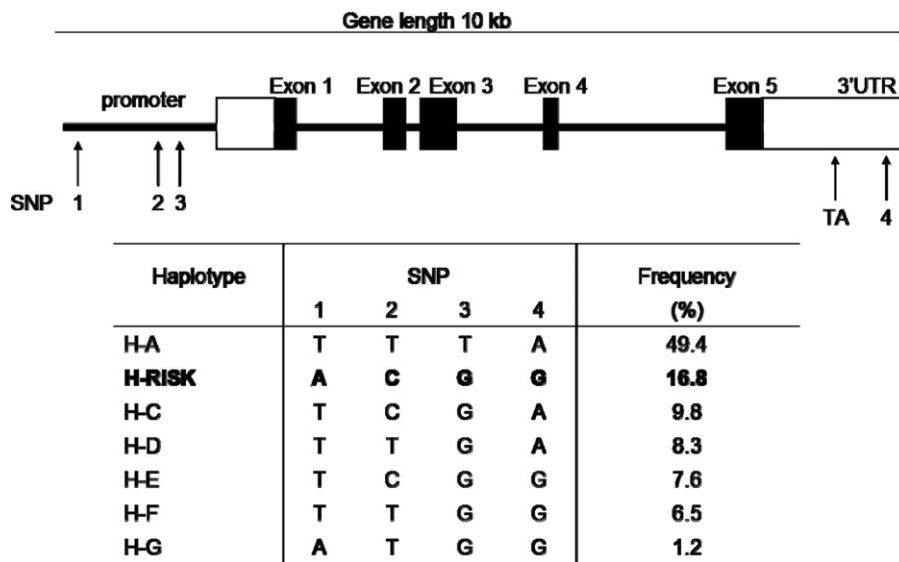
SNP	dbSNP reference <sup>a</sup>	Chromosomal coordinate <sup>b</sup>	Position <sup>c</sup>	Alleles MAJOR (minor)	Frequency <sup>d</sup> %
1	rs1800890	203337760	-3575	T (a)	18
2	rs1800896	203335292	-1082	T (c)	34
3	rs1800872	203334802	-592	T (g)	49
4	rs3024500	203329226	5009	A (g)	32

<sup>a</sup>As described in <http://www.ncbi.nlm.nih.gov/projects/snp>.

<sup>b</sup>Coordinate in chromosome 1 as described in Ensemble release v36, Dec 2005.

<sup>c</sup>SNPs designated according to the nucleotide position relative to the transcriptional starting site.

<sup>d</sup>Frequency of the minor allele in The Gambian population.



**Figure 1.** Haplotype structure at the *IL10* locus in the Gambian population. Haplotype frequencies in the general population are shown for each haplotype. Designation of SNPs refers to those from Supplementary Material, Table S1. TA refers to the rs3024496 SNP present in the transcript. H-RISK (*IL10*-3575A/*IL10*-1082C/*IL10*-592G/*IL10*+5009G) containing four haplotype-tagging SNPs significantly associated with trachomatous scarring and trichiasis (risk haplotype).

with a particular haplotype of *IL10* (here referred to as the risk haplotype – H-RISK), which we have shown to be associated with increased risk of scarring trachoma in Gambian subjects (5). In clinical studies, conjunctival expression of *IL10* is elevated during active trachomatous inflammation (11,12). The goal of this study was to test the hypothesis that the H-RISK acts to increase conjunctival *IL10* expression during chlamydial infection. We have used allele-specific transcript quantification (ASTQ) (13–15) to investigate the effects of the *IL10* H-RISK on *IL10* expression in a specific clinical context, i.e. at the site of infection, in the conjunctival epithelium of subjects with active trachoma.

## RESULTS

Table 1 and Figure 1 specify the single nucleotide polymorphisms (SNPs) that define the risk haplotype (H-RISK: *IL10*-3575A/*IL10*-1082C/*IL10*-592G/*IL10*+5009G), which in a previous Gambian case-control study was associated with increased risk of trichiasis and scarring trachoma (5).

The starting point of this study was to identify a genetic marker which could be used to distinguish *IL10* transcripts

arising from H-RISK or from other haplotypes. We sequenced transcribed regions of the *IL10* gene in 13 Gambian individuals, in whom 10 out of 26 chromosomes carried H-RISK. We identified an SNP in the 3'-untranslated region (3'-UTR) of the *IL10* transcript sequence (rs3024496) which was strongly associated with the H-RISK in the sequenced individuals (Fig. 2). If the major allele of rs3024496 is denoted as transcript allele 1 (TA1) and the minor allele TA2, then three out of three homozygotes for the H-RISK were homozygous for TA2; while five out of six individuals without the H-RISK were homozygous for TA1. In contrast, among 15 chromosomes with common haplotypes other than H-RISK, only four carried the TA2 allele.

The above findings indicated that the TA2 allele could serve as a genetic marker of *IL10* transcripts that arise from the H-RISK. To further assess this relationship, we typed SNP rs3024496 in the Gambian case-control study that has previously been described (5), comprising 651 individuals with scarring trachoma or trichiasis, plus a similar number of controls matched by age, sex and ethnicity (5). As shown in Table 2, the risk of long-term complications of trachoma increased with copy number of the TA2 allele, both for

Polymorphisms			# Copies of H-RISK												
Sequence	dbSNP reference <sup>a</sup>	Chromosomal coordinate <sup>b</sup>	TWO			ONE				NONE					
			case-3	control-1	case-2	case-6	case-5	case-4	case-7	control-13	control-12	control-9	control-10	control-11	control-8
GCG C/T ACC	rs6687786	203329615													
TCG G/A AGG	rs6697497	203329858													
GCT A/g CAA	rs3024498	203329924													
AGG A/g AAA	not available	203330056													
AGA A/g TAT	rs3024496	203330259													

**Figure 2.** Comparison of five SNP sites across the IL10 transcript in the panel of 13 sequenced Gambian individuals. Homozygotes for the common allele are shaded light-grey, minor allele black and heterozygotes dark-grey. White indicates low quality read at this particular site. The clinical status (case-# refers to the trichiasis phenotype and control-# to subjects with healthy eyes) and number of copies of the H-RISK for each individual is reported. The rs3024496 minor allele refers to the IL10 risk variant TA. Note: <sup>a</sup><http://www.ncbi.nlm.nih.gov/projects/SNP/>, <sup>b</sup>coordinate in chromosome 1 as described in Ensembl release v36. Dec 2005.

**Table 2.** Proportions of cases and controls with different TA (rs3024496) SNP-genotypes and with different number of copies of the risk haplotype (H-RISK). The magnitude of associations is estimated as odds ratios and 95% confidence intervals (OR, 95%CI)

Genotype	Scarring phenotype No. of genotype (%)		CLR <sup>a</sup> OR (95%CI)	P-value	Trichiasis phenotype No. of genotype (%)		CLR <sup>a</sup> OR (95%CI)	P-value
	Cases	Controls			Cases	Controls		
TA1/TA1	258 (39.6)	294 (44.2)	Reference	–	116 (37.8)	144 (45.6)	Reference	–
TA1/TA2	293 (45.0)	266 (40.0)	1.51 (1.22, 1.87)	<0.001	139 (45.3)	120 (38.0)	1.81 (1.22, 2.69)	0.003
TA2/TA2	89 (13.7)	70 (10.5)	2.02 (1.47, 2.78)	<0.001	44 (14.3)	26 (8.2)	2.57 (1.41, 4.69)	0.002
<i>No. of copies H-RISK</i>								
0	425 (65.4)	457 (68.8)	Reference	–	189 (60.8)	227 (70.9)	Reference	–
1	200 (30.8)	191 (28.8)	1.19 (0.93, 1.53)	0.158	110 (35.4)	87 (27.2)	1.72 (1.19, 2.49)	0.004
2	25 (3.8)	16 (2.4)	2.16 (1.07, 4.36)	0.032	12 (3.9)	6 (1.9)	2.63 (0.90, 7.70)	0.076

<sup>a</sup>Risk estimates from conditional logistic regression (CLR) analysis of matched case-control pairs for the TA SNP and the H-RISK in the Scarring and Trichiasis phenotypes.

scarring trachoma (odds ratio (OR) for trend = 1.45, 95%CI = 1.25–1.68, *P* for trend <0.001) and for the more severe trichiasis phenotype (OR for trend = 1.67, 95%CI = 1.27–2.19, *P* for trend < 0.001). This is very similar to the results obtained when the H-RISK was analysed on the same samples (Table 2).

Our starting hypothesis was that high levels of conjunctival IL-10 production contribute to chronic complications of ocular *C. trachomatis* infection. Having established that an *IL10* haplotype which is a risk factor for scarring trachoma and trichiasis produces transcripts marked by the TA2 allele, our hypothesis can be tested in the following way: in the conjunctiva of Chlamydia-infected individuals (drawn from the same population in which the genetic observation was made), are *IL10* transcripts bearing the TA2 allele expressed in greater abundance than those bearing the TA1 allele?

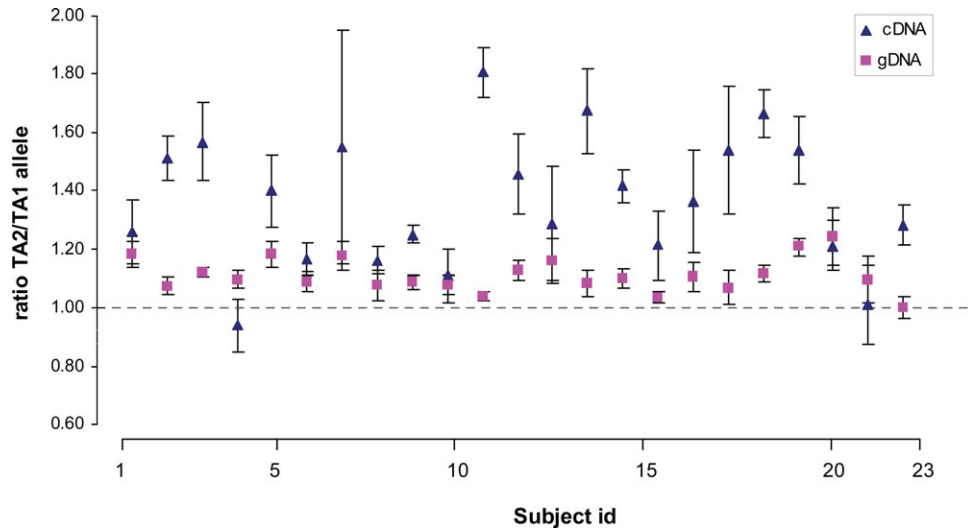
To address this question we identified 50 Gambian subjects with active trachoma. From each subject we obtained a sample of genomic DNA (gDNA) as well as a conjunctival swab from which we extracted RNA which we used to prepare cDNA. By genotyping the rs3024496 polymorphism in the gDNA sample we established that 23 of the 50 subjects were heterozygous, i.e. they had the capacity to make both the TA1 allele and the TA2 allele. In these 23 heterozygotes we used primer-extension/mass-spectrometry on cDNA to determine the TA1 : TA2 transcript ratio (i.e. the abundance of TA1 versus TA2 transcripts) in the conjunctival sample. To correct for any technical bias in allele calling, we used the same

method to determine the relative peak heights for TA1 and TA2 in gDNA from the same individual, and we used this information to normalize the TA1 : TA2 transcript ratio (a normalized TA1 : TA2 ratio of 1 implies that TA1 and TA2 are made in equal amounts).

Using the ASTQ method, we found that conjunctival *IL10* transcripts bearing the TA2 allele were expressed in greater abundance than those bearing the TA1 allele. One way of presenting the statistical significance of this observation is to consider the mass-spectrometry data prior to normalization: the TA2 : TA1 ratio was higher for cDNA than for gDNA in 20 of the 23 heterozygous individuals tested (*P* < 0.0001 Mann–Whitney U test, Fig. 3, Table 3). A more precise way of estimating the size of effect is to consider the mass-spectrometry data for cDNA after normalization using gDNA signal from the same individual: using this approach we estimate that the average ratio of TA2 : TA1 transcripts in the conjunctiva of heterozygous individuals infected with *C. trachomatis* is 1.23 (95% confidence interval (CI) 1.14–1.32; Fig. 3, Table 3).

## DISCUSSION

We have described a relative increase in *IL10* transcripts in the actively inflamed conjunctiva derived from the H-RISK allele in Gambian subjects with active trachoma. Our findings provide preliminary evidence that genetic factors affecting



**Figure 3.** Allele-specific expression of SNP rs3024496 in conjunctival RNA of 23 heterozygous individuals with active trachomatous inflammation. Primer-extension/mass-spectrometry was used to determine the relative abundance of the major (TA1) and minor (TA2) allele, expressed here as the TA2 : TA1 ratio, in gDNA (purple squares) and cDNA (blue triangles). Data shown are the mean and standard error of four independent PCR amplification reactions for cDNA (six for gDNA) assayed in duplicate by mass-spectrometry. The TA2 : TA1 ratio was higher for cDNA than for gDNA in 20 of the 23 heterozygous individuals tested ( $P < 0.0001$  by Mann–Whitney U test).

**Table 3.** Each individual's allele specific RT–PCR analysis for the rs3024496 marker. TA2 : TA1 cDNA and genomic DNA ratios are expressed as mean ratio and the standard error of the mean of the TA2 allele over the TA1 allele. Mann–Whitney and *t*-test statistics show whether there is significant differences between cDNA and gDNA allele ratios

Subject id	cDNA TA2 : TA1 ratio (mean)	SE	gDNA TA2 : TA1 ratio (mean)	SE	cDNA : gDNA ratio	Mann–Whitney <i>P</i> -value	<i>t</i> -test <i>P</i> -value
TF1	1.2564	0.110	1.1814	0.043	1.0635	0.3809	0.2795
TF2	1.5098	0.078	1.0749	0.028	1.4046	0.0047	0.0001
TF3	1.5690	0.132	1.1207	0.016	1.4000	0.5429	0.3707
TF4	0.9380	0.092	1.0957	0.030	0.8561	0.1286	0.9551
TF5	1.4000	0.124	1.1810	0.043	1.1854	0.0333	0.0427
TF6	1.1653	0.057	1.0880	0.035	1.0710	0.1286	0.1265
TF7	1.5498	0.403	1.1760	0.048	1.3179	0.3242	0.1216
TF8	1.1618	0.047	1.0757	0.053	1.0800	0.1762	0.1445
TF9	1.2500	0.032	1.0879	0.024	1.1490	0.0051	0.0009
TF10	1.1082	0.090	1.0787	0.034	1.0273	0.4524	0.3564
TF11	1.8058	0.086	1.0398	0.017	1.7367	0.0048	<0.0001
TF12	1.4560	0.135	1.1290	0.033	1.2896	0.0095	0.0105
TF13	1.2882	0.193	1.1599	0.076	1.1106	0.3048	0.2477
TF14	1.6743	0.147	1.0835	0.045	1.5453	0.0013	0.0007
TF15	1.4164	0.057	1.1010	0.033	1.2865	0.0048	0.0004
TF16	1.2129	0.117	1.0359	0.018	1.1709	0.1762	0.0505
TF17	1.3638	0.176	1.1050	0.048	1.2342	0.0857	0.0627
TF18	1.5409	0.220	1.0693	0.059	1.4410	0.0480	0.0129
TF19	1.6672	0.082	1.1144	0.028	1.4961	0.0048	<0.0001
TF20	1.5400	0.114	1.2079	0.030	1.2749	0.0086	0.0071
TF21	1.2105	0.086	1.2429	0.098	0.9739	0.5000	0.5867
TF22	1.0100	0.133	1.0961	0.081	0.9214	0.4520	0.7121
TF23	1.2832	0.069	0.9997	0.037	1.2836	0.0022	0.0072
<b>ALL</b>	<b>1.3642</b>	<b>0.046</b>	<b>1.1106</b>	<b>0.012</b>	<b>1.2313</b>	<b>&lt;0.0001</b>	<b>&lt;0.0001</b>

the risk of tissue damage following ocular *C. trachomatis* infection in humans are linked to allelic differences in *IL10* gene expression demonstrable at the site of infection and inflammation. The analysis suggests that the minor allele of rs3024496 (TA2) is a marker for the *IL10* transcript derived from the H-RISK; and that it therefore serves as a convenient

genetic marker to investigate allelic variation in the *cis*-regulation of *IL10* expression within heterozygous individuals. We show that in heterozygotes with active trachoma the ratio of TA2 : TA1 is increased in transcripts sampled in the conjunctiva relative to genomic DNA. This is the first time that allelic variation in the expression of a gene conferring suscep-



tibility to an infectious disease has been demonstrated at the site of infection in humans.

Because the risk allele is associated with enhanced *IL10* transcription, this study supports the hypothesis that high levels of conjunctival *IL10* expression in the active inflammatory stage of disease are deleterious for the disease process. There are several other lines of evidence which support deleterious effects of high IL-10 production on clearance of chlamydial infection, on protection from reinfection and on the subsequent development of scarring disease.

In human genital infection with *C.trachomatis*, an *IL10* haplotype correlating with low genital tract IL-10 production has been associated with decreased risk of recurrent infection (16). In a longitudinal study of genital chlamydial infection, *in vitro* IL-10 production by peripheral blood mononuclear cells (PBMCs) stimulated with recombinant chlamydial antigen was the greatest single risk factor for recurrent infection (17). A recent study of Finnish women with tubal factor infertility (TFI) evaluated *IL10-1082* genotypes previously linked to an increased risk of TFI (18). PBMCs from subjects with the *IL10-1082* CC genotype had increased IL-10 production, but also reduced interferon- $\gamma$  and lymphocyte proliferative responses to chlamydial antigen (19). The *IL10-1082* C allele is present in the trachoma H-RISK described here.

In experimental murine models of chlamydial infection, high IL-10 production delays clearance of the pathogen (20) and *IL10* knockout mice clear infection faster than genetically intact controls (21–23). Thus prematurely produced or excess IL-10 may exert immunoregulatory effects that handicap the development of a protective CD4+IFN $\gamma$  response to chlamydial infection (24). This parallels recent findings in leishmaniasis and tuberculosis suggesting that susceptibility to the intracellular pathogens *L.major* and *M.tuberculosis* is mediated by overproduction of IL-10 (22,25). We have found that the conjunctival expression of *IL10* and IFN $\gamma$  are well correlated during ocular chlamydial infection and the active phases of clinical disease (11), in keeping with induction of an IL-10 immunoregulatory response which may directly and indirectly contribute to fibrogenesis.

IL-10 contributes to the pathogenesis of fibrotic responses in numerous models of disease; for example in models of silicosis IL-10 has fibrogenic effects in the lungs of mice and *IL10* gene knockout mice are more resistant to the development of fibrosis (21,26). These and other studies have suggested that, in addition to its anti-inflammatory properties, IL-10 may have an important role in local matrix remodeling during healing and repair of inflammation-induced injury (27). These data support the hypothesis that the fibrotic phenotype of trachoma could be mediated, at least in part, by IL-10 over-expression.

There is evidence to suggest that variation at the *IL10* locus may be in part responsible for the differences in gene expression observed between diseased and healthy individuals in an increasing number of diseases (including systemic lupus, asthma and meningococcal disease) (18,28–33). However, identification of regulatory polymorphisms that correlate with these differences has been unsuccessful to date. There are difficulties in characterizing the relationship between disease susceptibility and genetic variability in gene

regulation. Allelic variation in gene regulation is thought to be highly specific for tissue and for biological context (34), so findings from experimental animal models or isolated tissue-specific cell lines such as PBMCs from healthy individuals may be an unreliable indicator of what happens at the site of disease in living human subjects. Our findings demonstrate the potential for ASTQ to serve as a powerful tool linking genetic epidemiology to functional analysis of gene expression.

It remains to be determined whether the TA 3'-UTR SNP is directly responsible for the functional effects detected in this work, or simply a marker for an unknown functional variant, but we can be sure that the functional effect is *cis*-regulatory because it was identified by direct comparison of the TA1 and TA2 alleles in heterozygous individuals. Very similar risk effects on disease were observed for this marker and the previously genotyped haplotype tagging SNPs located in the distal promoter and 5'-UTR of the *IL10* gene (5). The presence of these risk alleles in the H-RISK, and the closeness of the single-marker and haplotype risk effects, suggest that further fine mapping efforts in *IL10* and the neighbouring functionally-related genes is likely to identify the causal SNP(s).

We have used ASTQ to focus on *cis*-regulatory effects at the site of infection to test the hypothesis that the trachoma *IL10* H-RISK acts to increase conjunctival *IL10* expression during chlamydial infection. By comparing transcript levels in heterozygotes, rather than measuring differences in gene expression or IL-10 levels between individuals, we have avoided confounding by environmental factors, one can be confident that differences in transcription are due to the H-RISK itself. By testing *cis*-regulatory mechanisms in the conjunctival epithelium of subjects in trachoma endemic populations, we have been able to relate the *IL10* H-RISK *cis*-regulatory effects to risk of severe clinical outcome.

## MATERIALS AND METHODS

### Subjects for sequencing

DNA from six subjects with trichiasis and seven controls with normal eyelids from the association study was screened for variants in the transcribed region of *IL10* to determine if the haplotype could be 'tagged' by variants on the transcript.

### Sequencing

Amplification of *IL10* exons was carried out using M13-tailed oligonucleotides and the Expand High Fidelity PCR system (Roche) following the manufacturer's recommendations (Supplementary Material, Table S1 for forward and reverse primers). Each of the amplified products was sequenced using the ABI BigDye Primer v3.0 Cycle Sequencing Ready Reaction kit and an ABI PRISM<sup>®</sup> 3700 DNA Analyser (Applied Biosystems, Warrington, UK) following the manufacturer recommendations. PolyPhred (<http://droog.gs.washington.edu/PolyPhred.html>) was used for sequence assembly and analysis.

**SNP genotyping**

The SNP rs3024496 identified on the *IL10* transcript was genotyped in the subjects from the case-control association study previously described (9). Homogenous MassEXTEND<sup>®</sup> Assay (Sequenom<sup>®</sup>, Hamburg, Germany) matrix-assisted laser-desorption/ionization time-of-flight mass spectrometry (hME-MS) was used as previously described (5). The SNP was typed with respect to the positive chromosome strand. The first-round PCR product was generated using the hME-tailed oligonucleotides:

Forward: 5'-ACGTTGGATGTCGTTACAGAGAAGCTCAG-3',

Reverse: 5'-ACGTTGGATGCCCTTGAGAAACCTTATG-3'.

The extension reaction used an ACG tri-terminator mix and the oligonucleotide 5'-TGAGAAACCTTATTGTACCTCTCT-3'. The major and minor alleles of rs3024496 are referred to as TA1 and TA2, respectively.

**Statistical analysis of association study**

Stepwise conditional logistic regression (CLR) analysis was used to evaluate the effects of the H-RISK and the SNP TA on the risk of scarring in the association study, and assess the dependence between them. All analyses were performed using STATA software (v8.0, <http://www.stata.com>).

**Subjects with active trachoma selected for allele-specific PCR**

Fifty new cases of active trachomatous inflammation were recruited from the same trachoma-endemic communities as those of the association for studies of conjunctival *IL10* expression. Fifty percent of the subjects were female and the mean age was 11 years-old (ranging from 2 to 13 years-old). Most subjects belonged to two common ethnic groups in the Gambia: Manjago and Mandinka.

For each subject an eye swab, and a buccal-brush were collected for RNA and DNA extraction, respectively [eye swabs were collected in 'RNA later' reagent (Ambion) and stored at -70°C prior to processing]. Following genotyping 23 subjects determined to be heterozygous for the risk allele were selected for further investigation and ASTQ.

**Allele-specific RT-PCR by hME-MS**

Total RNA was isolated from conjunctival swabs using the RNeasy Micro kit (Qiagen Ltd, Crawley, UK) as described previously (12). The quantity and quality of total RNA was assessed using the Agilent Bioanalyser 2100 (Agilent) prior to further processing to exclude from the analysis samples regarded as degraded/low quality. Each sample was converted to cDNA using 220 ng of total RNA and SuperScript II RT (Invitrogen Ltd, Paisley, UK) with random primers. A further 120 ng of total RNA were processed without RT present as negative controls to check for genomic DNA contamination. Briefly, total RNA was mixed with 200 ng of random primers (3 µg/ml) and 10 mM dNTPs each to a total

volume of 12 µl. The RNA/primer mix was denatured at 65°C for 5 min and cooled on ice followed by addition of 4 µl 5× first-strand buffer, 2 µl of 0.1M DTT, 40U of RNase-OUT (Invitrogen Ltd) with or without 200U of SuperScript II RT enzyme. The mixture was incubated at 25°C for 10 min and 42°C for 50 min. A final inactivation step was carried out at 70°C for 15 min. Two units of *E.coli* RNase H were added and incubated at 37°C for 20 min to remove RNA complementary to the cDNA. After completion of the first-strand synthesis, single-strand complementary DNA (scDNA) was purified using the Qiaquick PCR purification kit (Qiagen Ltd) and eluted with 50 µl of water. The volume of the samples was adjusted to 15 µl by vacuum drying (RT negative control volumes were vacuum reduced to 8 µl to compensate for the starting amount of total RNA). The allele expression ratio (AER) of SNP rs3024496 was measured using the standard hME-MS method as described above for genotyping, except that for each scDNA sample, 4×10 µl first-round reactions were prepared and two extension reactions were run from each first-round reaction. This gave eight genotype results per scDNA sample. Similarly, 12 technical replicates for the respective genomic DNA were run in parallel on the same hME chip. The relative abundance of allele-specific scDNA can be calculated using the Sequenom<sup>®</sup> Typer v 3.1 software using the area below each specific-allele peak. Using this information, AER at rs3024496 was inferred for scDNA and gDNA in each sample. The gDNA sample ratio also identifies any allele-ratio skew inherent to the SNP being typed; this ratio was then used to adjust the scDNA ratios. Means and standard errors were calculated for each of the four first-round reactions from the allele ratios of respective four extension reactions. These were combined to form a grand mean and standard error for the given sample.

**Analysis of transcript data**

Analysis was performed using the STATA v8 package. Data were summarized as mean with standard errors, geometric means with 95% CIs or medians with an interquartile range dependent on the distribution of the data. Student's paired *t*-test was used to test for significance of differences in mean ratios between mRNA and gDNA samples when data was normally distributed. The Mann-Whitney test was used to test for significance in difference in mean ratios when the data did not fit a normal distribution.

**SUPPLEMENTARY MATERIAL**

Supplementary Material is available at HMG Online.

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*Conflict of Interest statement.* The authors declare that they have no competing financial interests.

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## REFERENCES

- Mariotti, S.P. (2004) New steps toward eliminating blinding trachoma. *N. Engl. J. Med.*, **351**, 2004–2007.
- Mabey, D.C., Solomon, A.W. and Foster, A. (2003) Trachoma. *Lancet*, **362**, 223–229.
- Fenton, K.A. and Lowndes, C.M. (2004) Recent trends in the epidemiology of sexually transmitted infections in the European Union. *Sex Transm. Infect.*, **80**, 255–263.
- Mabey, D.C.W. (1992) The epidemiology and pathogenesis of trachoma. *Rev. Med. Microbiol.*, **3**, 112–119.
- Natividad, A., Wilson, J., Koch, O., Holland, M.J., Rockett, K., Faal, N., Jallow, O., Joof, H.M., Burton, M.J., Alexander, N.D. *et al.* (2005) Risk of trachomatous scarring and trichiasis in Gambians varies with SNP haplotypes at the interferon-gamma and interleukin-10 loci. *Genes Immun.*, **6**, 332–340.
- Natividad, A., Cooke, G., Holland, M.J., Burton, M.J., Joof, H.M., Rockett, K., Kwiatkowski, D.P., Mabey, D.C. and Bailey, R.L. (2006) A coding polymorphism in matrix metalloproteinase 9 reduces risk of scarring sequelae of ocular Chlamydia trachomatis infection. *BMC Med. Genet.*, **7**, 40.
- Natividad, A., Hanchard, N., Holland, M.J., Mahdi, O.S., Diakite, M., Rockett, K., Jallow, O., Joof, H.M., Kwiatkowski, D.P., Mabey, D.C. *et al.* (2007) Genetic variation at the TNF locus and the risk of severe sequelae of ocular Chlamydia trachomatis infection in Gambians. *Genes Immun.*, **8**, 288–295.
- Mozzato-Chamay, N., Corbett, E.L., Bailey, R.L., Mabey, D.C., Raynes, J. and Conway, D.J. (2001) Polymorphisms in the IkappaB-alpha promoter region and risk of diseases involving inflammation and fibrosis. *Genes Immun.*, **2**, 153–155.
- Mozzato-Chamay, N., Mahdi, O.S., Jallow, O., Mabey, D.C., Bailey, R.L. and Conway, D.J. (2000) Polymorphisms in candidate genes and risk of scarring trachoma in a Chlamydia trachomatis-endemic population. *J. Infect. Dis.*, **182**, 1545–1548.
- Conway, D.J., Holland, M.J., Bailey, R.L., Campbell, A.E., Mahdi, O.S., Jennings, R., Mbena, E. and Mabey, D.C. (1997) Scarring trachoma is associated with polymorphism in the tumor necrosis factor alpha (TNF-alpha) gene promoter and with elevated TNF-alpha levels in tear fluid. *Infect. Immun.*, **65**, 1003–1006.
- Faal, N., Bailey, R.L., Sarr, I., Joof, H., Mabey, D.C. and Holland, M.J. (2005) Temporal cytokine gene expression patterns in subjects with trachoma identify distinct conjunctival responses associated with infection. *Clin. Exp. Immunol.*, **142**, 347–353.
- Burton, M.J., Bailey, R.L., Jeffries, D., Mabey, D.C. and Holland, M.J. (2004) Cytokine and fibrogenic gene expression in the conjunctivas of subjects from a Gambian community where trachoma is endemic. *Infect. Immun.*, **72**, 7352–7356.
- Yan, H., Yuan, W., Velculescu, V.E., Vogelstein, B. and Kinzler, K.W. (2002) Allelic variation in human gene expression. *Science*, **297**, 1143.
- Pastinen, T. and Hudson, T.J. (2004) Cis-acting regulatory variation in the human genome. *Science*, **306**, 647–650.
- Knight, J.C., Keating, B.J., Rockett, K.A. and Kwiatkowski, D.P. (2003) In vivo characterization of regulatory polymorphisms by allele-specific quantification of RNA polymerase loading. *Nat. Genet.*, **33**, 469–475.
- Wang, C., Tang, J., Geisler, W.M., Crowley-Nowick, P.A., Wilson, C.M. and Kaslow, R.A. (2005) Human leukocyte antigen and cytokine gene variants as predictors of recurrent Chlamydia trachomatis infection in high-risk adolescents. *J. Infect. Dis.*, **191**, 1084–1092.
- Cohen, C.R., Koochesfahani, K.M., Meier, A.S., Shen, C., Karunakaran, K., Ondondo, B., Kinyari, T., Mugo, N.R., Nguti, R. and Brunham, R.C. (2005) Immunoepidemiologic profile of Chlamydia trachomatis infection: importance of heat-shock protein 60 and interferon-gamma. *J. Infect. Dis.*, **192**, 591–599.
- Kinnunen, A.H., Surcel, H.M., Lehtinen, M., Karhukorpi, J., Tiitinen, A., Halttunen, M., Bloigu, A., Morrison, R.P., Karttunen, R. and Paavonen, J. (2002) HLA DQ alleles and interleukin-10 polymorphism associated with Chlamydia trachomatis-related tubal factor infertility: a case-control study. *Hum. Reprod.*, **17**, 2073–2078.
- Ohman, H., Tiitinen, A., Halttunen, M., Birkelund, S., Christiansen, G., Koskela, P., Lehtinen, M., Paavonen, J. and Surcel, H.M. (2006) IL-10 polymorphism and cell-mediated immune response to Chlamydia trachomatis. *Genes Immun.*, **7**, 243–249.
- Yang, X., Gartner, J., Zhu, L., Wang, S. and Brunham, R.C. (1999) IL-10 gene knockout mice show enhanced Th1-like protective immunity and absent granuloma formation following Chlamydia trachomatis lung infection. *J. Immunol.*, **162**, 1010–1017.
- Yang, X., HayGlass, K.T. and Brunham, R.C. (1996) Genetically determined differences in IL-10 and IFN-gamma responses correlate with clearance of Chlamydia trachomatis mouse pneumonitis infection. *J. Immunol.*, **156**, 4338–4344.
- Redpath, S., Ghazal, P. and Gascoigne, N.R. (2001) Hijacking and exploitation of IL-10 by intracellular pathogens. *Trends Microbiol.*, **9**, 86–92.
- Igietseme, J.U., Ananaba, G.A., Bolier, J., Bowers, S., Moore, T., Belay, T., Eko, F.O., Lyn, D. and Black, C.M. (2000) Suppression of endogenous IL-10 gene expression in dendritic cells enhances antigen presentation for specific Th1 induction: potential for cellular vaccine development. *J. Immunol.*, **164**, 4212–4219.
- McMahon-Pratt, D. and Alexander, J. (2004) Does the Leishmania major paradigm of pathogenesis and protection hold for New World cutaneous leishmaniases or the visceral disease? *Immunol. Rev.*, **201**, 206–224.
- Kane, M.M. and Mosser, D.M. (2001) The role of IL-10 in promoting disease progression in leishmaniasis. *J. Immunol.*, **166**, 1141–1147.
- Cowles, C.R., Hirschhorn, J.N., Altshuler, D. and Lander, E.S. (2002) Detection of regulatory variation in mouse genes. *Nat. Genet.*, **32**, 432–437.
- Barbarin, V., Xing, Z., Delos, M., Lison, D. and Huaux, F. (2005) Pulmonary overexpression of IL-10 augments lung fibrosis and Th2 responses induced by silica particles. *Am. J. Physiol. Lung Cell Mol. Physiol.*, **288**, L841–L848.
- Lee, C.G., Homer, R.J., Cohn, L., Link, H., Jung, S., Craft, J.E., Graham, B.S., Johnson, T.R. and Elias, J.A. (2002) Transgenic overexpression of interleukin (IL)-10 in the lung causes mucus metaplasia, tissue inflammation, and airway remodeling via IL-13-dependent and -independent pathways. *J. Biol. Chem.*, **277**, 35466–35474.
- Lazarus, M., Hajeer, A.H., Turner, D., Sinnott, P., Worthington, J., Ollier, W.E. and Hutchinson, I.V. (1997) Genetic variation in the interleukin 10 gene promoter and systemic lupus erythematosus. *J. Rheumatol.*, **24**, 2314–2317.
- Gibson, A.W., Edberg, J.C., Wu, J., Westendorp, R.G., Huizinga, T.W. and Kimberly, R.P. (2001) Novel single nucleotide polymorphisms in the distal IL-10 promoter affect IL-10 production and enhance the risk of systemic lupus erythematosus. *J. Immunol.*, **166**, 3915–3922.
- Crawley, E., Isenberg, D., Woo, P. and Kay, R. (1999) Interleukin-10 promoter polymorphism and lupus nephritis: comment on the article by Mok *et al.* *Arthritis Rheum.*, **42**, 590–593.
- Lim, S., Crawley, E., Woo, P. and Barnes, P.J. (1998) Haplotype associated with low interleukin-10 production in patients with severe asthma. *Lancet*, **352**, 113.
- Llorente, L., Richaud-Patin, Y., Couderc, J., Alarcon-Segovia, D., Ruiz-Soto, R., Alcocer-Castillejos, N., Alcocer-Varela, J., Granados, J., Bahena, S., Galanaud, P. *et al.* (1997) Dysregulation of interleukin-10 production in relatives of patients with systemic lupus erythematosus. *Arthritis Rheum.*, **40**, 1429–1435.
- Westendorp, R.G., Langermans, J.A., Huizinga, T.W., Elouali, A.H., Verweij, C.L., Boomsma, D.I., Vandenbroucke, J.P. and Vandenbroucke, J.P. (1997) Genetic influence on cytokine production and fatal meningococcal disease. *Lancet*, **349**, 170–173.