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Conjunctival Transcriptome in Scarring Trachoma▽†

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Trachoma is a poorly understood immunofibrogenic disease process, initiated by *Chlamydia trachomatis*. Differences in conjunctival gene expression profiles between Ethiopians with trachomatous trichiasis (with [TTI] or without [TT] inflammation) and controls (C) were investigated to identify relevant host responses. Tarsal conjunctival swab samples were collected for RNA isolation and *C. trachomatis* PCR. Transcriptome-wide microarray experiments were conducted on 42 samples (TTI, $n = 13$; TT, $n = 15$; C, $n = 14$). Specific results were confirmed by using multiplex quantitative reverse transcription-PCR for 16 mRNA targets in an independent collection of case-control samples: 386 case-control pairs (TTI, $n = 244$; TT, $n = 142$; C, $n = 386$). The gene expression profiles of cases were consistent with squamous metaplasia (keratins, SPRR), proinflammatory cytokine production (*IL1*β, *CXCL5*, and *S100A7*), and tissue remodeling (*MMP7*, *MMP9*, *MMP12*, and *HAS3*). There was no difference in the level of *IFN*γ between cases and controls. However, cases had increased *INDO*, *NOS2A*, and *IL13RA2* and reduced *IL13*. *C. trachomatis* was detected in 1/772. Cases show evidence of ongoing inflammation and tissue remodeling, which were more marked where clinical inflammation was also present. Significantly, these processes appear to be active in the absence of current *C. trachomatis* infection. There was limited evidence of a T_H1 response (*INDO* and *NOS2A*) and no association between a T_H2 response and cases. The epithelium appears to be actively involved in late cicatricial stages of trachoma through the production of proinflammatory factors (*IL1*β, *CXCL5*, and *S100A7*). Longitudinal studies are needed to investigate which etiological factors and pathways are associated with progressive scarring and whether simply controlling chlamydial infection will halt progression in people with established cicatricial disease.

Trachoma is the leading infectious cause of blindness worldwide (57). The disease process starts in early childhood with recurrent conjunctival infection by the bacterium *Chlamydia trachomatis*. This triggers a florid chronic inflammatory response characterized clinically by papillary hypertrophy and lymphoid follicles (active trachoma). In later life, after repeated episodes of infection and inflammation, scar tissue begins to accumulate within the conjunctiva, particularly that lining the upper eyelid. This causes eyelid distortion leading to the rubbing of lashes on the cornea (trichiasis). Ultimately, blinding corneal opacification develops.

The tissue damage in trachoma is primarily due to the human immune response rather than direct damage by the pathogen. Chlamydial infection is limited to the superficial conjunctival epithelium, while the inflammatory response extends into the deeper subepithelial layers (23). Moreover, clinical inflammation is often found in the absence of detectable infection (11). Studies of *C. trachomatis* infection in a monkey model demonstrated that after a primary infection the inflammatory phenotype could be induced by inoculating the conjunctiva with the *C. trachomatis* HSP60 antigen (66). There are some data on the immunological response in early active trachoma; however, there is limited information about the cicatricial stage (10, 28, 30, 44, 45). It is unknown which fibrogenic pathways are important to this disease process and what drives them. There are some data which show that individuals who have repeated severe inflammation in childhood are more likely to develop scarring complications in later life (16, 71). However, there are no longitudinal data that directly demonstrate that repeated *C. trachomatis* infection is needed for progressive scarring in later life, although scarring complications are generally more frequent and severe in regions where there is a high prevalence of active disease or *C. trachomatis* infection.

Previous work on trachoma and analogous disease processes has suggested a number of potential mechanisms for the development of scarring. It was suggested that a predominantly T_H1 type response may be protective and that a predominantly T_H2 response may be associated with the development of scarring (28). In addition, there is mounting evidence for the importance of T_H2 responses in other infectious diseases that produce scarring such as schistosomiasis, which may be mediated by interleukin-13 (IL-13) and alternative macrophage activation (73). There are also some data indicating that factors such as the matrix metalloproteinases may be involved in the tissue remodeling process in trachoma (9, 10, 21, 30).

Azithromycin can be effective in controlling chlamydial infection in communities where coverage rates are high (61).

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However, there are significant obstacles to its effective regular long-term delivery. Similarly, improved control of genital chlamydial infection can be achieved through screening programs; however, these are expensive, and uptake is low. Therefore, an antichlamydial vaccine would be useful (64). Trachoma vaccine trials in the 1960s induced short-lived, serovar-specific protection with whole-organism vaccines (25, 69). Furthermore, in some trials vaccination was thought to be associated with more severe disease, suggesting immunopathological tissue damage (63). Therefore, a critical issue is whether an antichlamydial response can be induced without provoking fibrosis. A better understanding of how chlamydial infections damage tissue and cause fibrosis is necessary for the development of a safe vaccine that will prime the immune system to produce nonfibrogenic responses.

To further our understanding of scarring trachom, we investigated the conjunctival gene expression profile in a group of Ethiopian individuals with established trachomatous scarring and trichiasis (with or without inflammation). These cases were compared to control subjects from the same communities. We initially performed microarray analysis on a limited set of samples. Subsequently, quantitative reverse transcription-PCR (RT-PCR) was used to validate microarray findings and explore specific hypotheses in a large case-control series.

MATERIALS AND METHODS

Ethical permission. This study was approved by the National Health Research Ethics Review Committee, Ministry of Science and Technology of Ethiopia; the London School of Hygiene and Tropical Medicine Ethics Committee; and the Emory University Institutional Review Board. Informed consent was obtained prior to enrolment of each subject. This study adhered to the tenets of the Declaration of Helsinki.

Study participants. Individuals with trachomatous trichiasis were recruited during a surgical campaign in Amhara Region, Ethiopia. Control subjects, who did not have either trichiasis or significant conjunctival scarring, were recruited from the same communities as the cases. We recruited three groups of subjects (i) patients with trichiasis and inflammation (TTI), (ii) patients with trichiasis but no conjunctival inflammation (TT), and (iii) controls without trichiasis or significant conjunctival scarring (C). A much larger number of trichiasis cases (both TTI and TT) and matched controls were recruited for real-time PCR assessment of gene expression. Where possible, controls were of the same sex, similar age ($\pm$10 years) and came from the same community as their matched case.

Clinical assessment and sample collection. All participants were examined for clinical signs of trachoma, graded using 2.5× binocular loupes according to the detailed World Health Organization (WHO) trachoma grading system (15). Trichiasis was defined as lashes touching the eye or clear evidence of epilation of trichiatic lashes. Conjunctival inflammation was considered significant if there were prominent papillae and/or haziness of the upper tarsal blood vessels (P_2/P_3) (15). Conjunctival scarring was considered significant if there were easily visible moderate or severe scars (C_2/C_3) on the upper tarsal conjunctiva (15). The conjunctiva was anesthetized with preservative-free proxymetacaine 0.5% eye drops (Minims; Chauvin Pharmaceuticals). Two conjunctival swab samples were collected from the upper tarsal conjunctival surface (Dacron polyester-tipped swab; Hardwood Products Company). The first was collected for RNA isolation and placed directly into a tube containing 0.3 ml of RNA*later* (Ambion). The second swab was collected for *C. trachomatis* PCR and placed in a dry tube. Samples were kept on ice packs until frozen later the same day at −20°C.

Microarray experiments. RNA was extracted from swab samples by using an RNeasy Micro kit (Qiagen, Crawley, United Kingdom) according to the manufacturer's instructions. Total RNA was assessed for concentration and quality on a NanoDrop ND-1000 (Thermo Fisher Scientific, Loughborough, United Kingdom) and a Bioanalyser (Agilent Technologies, Queensferry, United Kingdom). Microarray experiments were performed at Cambridge Genomic Services, University of Cambridge, using the HumanWG-6 v3.0 Expression BeadChip (Illumina, San Diego, CA) according to the manufacturer's instructions. Briefly, RNA underwent linear amplification using the Illumina TotalPrep RNA amplification kit (Ambion, Warrington, United Kingdom) according to the manufacturer's instructions. The cRNA was checked on a NanoDrop and Bioanalyser for concentration and to verify the success of amplification. If the concentration was below 150 ng/μl, this was increased by using a SpeedVac (Thermo Fisher Scientific). cRNA was hybridized to the HumanWG-6 BeadChip overnight. The next day the BeadChips were washed and stained. Finally, the chips were scanned by using a bead array reader (Illumina).

Quantitative RT-PCR. The abundance of 16 selected transcripts was estimated by quantitative RT-PCR. Gene targets (see Table 6) were chosen to address the hypothesized roles of T_H1/T_H2 balance (*IFNG*, *IL13*, and *1L13RA2*), classical and alternative macrophage activation (*NOS2A* and *ARG1*), and matrix metalloproteinases (*MMP7*, *MMP9*, *MMP12*, and *HAS3*) in the fibrotic process. In addition, four targets (*S100A7* [psoriasin], *CXCL5*, *HAS3*, and *IL19*) identified in the microarray experiment as having marked increase in expression in cases, and the potential to be important in this disease process, were also selected for quantitative RT-PCR estimation.

Total RNA was extracted from the swab samples using the RNeasy Micro kit (Qiagen). Reverse transcription was performed by using a QuantiTect reverse transcription kit (Qiagen) according to the manufacturer's instructions. This contains a mix of oligo(dT) and random primers. Multiplex real-time quantitative PCR was performed on a Rotor-Gene 6000 (Corbett Research, Cambridge, United Kingdom) using the QuantiTect Multiplex NoROX kit (Qiagen), according to the manufacturer's instructions. Multiplex assays of up to four separate targets (including *HPRT-1* as the reference gene) were designed by Sigma Life Science (Sigma Primer and Probe Design Service) using Beacon Designer 7.60 (Premier Biosoft International, Palo Alto, CA). The thermal cycle protocol used the following conditions: 95°C for 15 min, followed by 45 cycles of first denaturation at 94°C for 30 s and then annealing and extension at 60°C for 30 s. Fluorescence data was acquired at the end of each cycle. The relative efficiency of the component reactions was assessed using standards containing all targets in a sequence of 10-fold serial dilutions. Reactions were performed in duplicate, in a total volume of 25 μl, which contained 2 μl of sample or standard. Probe and primer sequences are available on request.

***C. trachomatis* detection.** Conjunctival swab samples collected from the subjects in the RT-PCR case-control study were tested for *C. trachomatis* DNA by using a PCR-based assay (Amplicor CT/NG Test; Roche) with previously described modifications (11). We did not collect DNA swabs from individuals in the microarray component of the study.

Microarray analysis. Quality control checks on the microarray data were made using GenomeStudio (Illumina). Sample independent controls were used to assess the success of the microarray process (hybridization, stringency, biotin control, and negative controls). Sample dependent controls examined the intensities of reference genes and the average intensity of all probes on each strip of the array. The data were transferred to Lumi (www.bioconductor.org), an open-source software package written in R (www.r-project.org), for the analysis of Illumina microarray data (19). Additional checks on the correlation between replicates and the intensity distribution were made. Nonsupervised clustering was performed to identify potential outliers. Transcripts were selected for analysis if they were expressed in at least one sample and they were significantly different from the negative controls at the 1% significance level. The data were transformed using the variance stabilization transformation and then normalized using quantile normalization in Lumi (40). Normalized data were examined by using the open-source R-based package LIMMA (www.bioconductor.org) in three pairwise comparisons: TTI versus C, TT versus C, and TTI versus TT (60). We corrected for multiple testing by using the Benjamini-Hochberg method for adjusting the *P* value and thereby controlling the false discovery rate (3). Gene enrichment analysis of gene ontology terms and pathways were performed for the three pairwise comparisons using DAVID (v6.7; http://david.abcc.ncifcrf.gov) and GeneGO (GeneGO, St. Joseph, MI) (18, 32). Enriched gene ontology terms were identified by assessing the proportion of genes in a particular grouping that were either upregulated (fold change, >1.5) or downregulated (fold change, <0.66) with an adjusted *P* value of <0.05.

The microarray data discussed in this publication have been deposited in NCBI's Gene Expression Omnibus and are accessible through GEO Series accession number GSE23705 (http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE23705).

Quantitative RT-PCR analysis. The transcript abundances for genes of interest were standardized relative to that of *HPRT1* in the same reaction using the $\Delta\Delta C_T$ method and were successfully normalized by $\log_{10}$ transformation (41). An overall comparison of the mean values across the three different clinical categories was made using one-way analysis of variance. Paired comparisons between clinical categories were made using either paired or unpaired Student *t* tests as appropriate. Bonferroni correction for 48 multiple comparisons gave a critical significance threshold value of $P = 0.001$.

TABLE 1. Demographic characteristics of subjects in microarray study grouped by clinical phenotype[a]

Parameter	C (n = 14)	TTI (n = 13)	TT (n = 15)
No. of male subjects (%)	7 (50)	2 (15)	6 (40)
No. of female subjects (%)	7 (50)	11 (85)	9 (60)
Mean age in yr (95% CI)	43.5 (36.3–52.2)	40.8 (32.2–51.6)	56.1 (50.0–93.0)

[a] Subject group: TTI, trichiasis with inflammation; TT, trichiasis without inflammation; C, control; n, number of subjects.

RESULTS

Study participants. All participants were of Amharan Ethiopian ethnicity. We recruited and collected samples from 45 individuals for the microarray study. The mean RNA concentration in the extract was 76 ng/μl (standard deviation [SD] = 23; range, 26 to 125 ng/μl). Three samples were not included in the microarray experiment because their RNA concentration was insufficient (2 TTI and 1 C). The numbers, age, and gender profiles of the three clinical groups (TTI, TT, and C) are shown in Table 1. None of the control subjects in the microarray experiment had any visible conjunctival scarring.

For the quantitative RT-PCR component of the present study we recruited 772 individuals: 386 trichiasis cases (TT, n = 142; TTI, n = 244) and 386 controls, whose demographic and clinical characteristics are shown in Table 2. The severity of endemic trachoma in this Ethiopian population is such that there are relatively few adults who are totally free of any tarsal conjunctival scarring. Therefore, to meet other matching criteria for controls (location, age, and sex), we accepted controls with minor degrees of conjunctival scarring (WHO C_1 or mild C_2).(15). The vast majority of individuals (762 of 772) in the present study lived in districts that have been mass treated with azithromycin for trachoma control during the year prior to surgery with an average population coverage of 77% (The Carter Center, Ethiopia). There had been no treatments during the 3 months prior to when sampling started.

Microarray quality control and data selection. The microarray internal sample-independent quality control tests all yielded "good" results. Reference genes performed well, indicating that there was no template degradation. Of the 44,803 probe sets that can be detected by the Human WG-6 microarray, 17,214 were selected for normalization and analysis.

Global gene expression and clinical state. To assess the extent to which global gene expression profiles could distinguish the clinical groups, a 42×42 matrix of the Pearson correlation coefficients of global gene expression for each pair of subjects was constructed (Fig. 1). Each row and column corresponds to an individual subject and the entry in the ith row and jth column being the Pearson correlation r_{ij} between the global expression profiles of individuals i and j. The matrix was reordered so that subjects whose profiles were most similar were placed in adjacent rows and columns and a hierarchical tree of similarity constructed (Fig. 1). This hierarchical tree had two main branches. All individuals with TTI were found in branch 1, and all normal controls were in branch 2. The cases with TT (without inflammation) were found in about equal proportion in both branches. Within branch 1 the TTI and the TT cases mostly segregated into separate clusters. Similarly, in branch 2 the controls and the TT cases generally segregated.

TABLE 2. Demographic and clinical characteristics of cases and controls in the quantitative RT-PCR study[a]

Parameter	Cases (n = 386)		Controls (n = 386)	
	No.	%	No.	%
No. of female subjects	290	75.1	269	69.7
No. of subjects at age:				
<40 yr	90	23.3	107	27.7
40–49 yr	102	26.4	99	25.6
50–59 yr	94	24.4	84	21.8
≥60 yr	100	25.9	96	24.9
Mean (95% CI)[b]	49.6	48.2–51.0	48.0	46.6–49.4
Mean BMI[c] (95% CI)	19.7	19.4–19.9	20.5	20.1–20.7
Trichiasis (no. of lashes)				
None			386	100
None (epilating)	60	15.5		
1–4	128	33.2		
5–9	115	29.8		
10–19	44	11.4		
≥20	39	10.1		
Mean (95% CI)	8.8	7.3–10.2		
No. of subjects with conjunctival inflammation				
P0	29	7.5	242	62.7
P1	113	29.3	136	35.2
P2	187	48.4	8	2.1
P3	57	14.8	0	0.0
No. of subjects with conjunctival scarring				
C0			105	27.2
C1	4	1.0	235	60.9
C2	239	61.9	46	11.9
C3	143	37.1		

[a] The percent values represent the percent total number. Range values indicate 95% CI values.
[b] Student t test, P = 0.10.
[c] Body mass index (BMI) data were available on 384 cases and 240 controls (Student t test, P = 0.0001).

Differentially regulated genes in different clinical states. For all 17,214 probe sets detected by microarray and selected for analysis expression levels were compared between the clinical groups: TTI versus C, TT versus C, and TTI versus TT. Transcripts found to be at an increased or a decreased abundance relative to the C group, or to the TT group in the case of TTI versus TT, were filtered with different adjusted P value thresholds (Table 3). The numbers of transcripts unique to each comparison with a >2-fold increase or decrease in abundance are shown in Fig. 2. In this Venn diagram, the origins of a selection of potentially interesting transcripts are indicated. The most numerous differences were between TTI and C, with less differential regulation occurring between TT and C and between TTI and TT cases. Selected transcripts of interest identified by microarray analysis are shown in Table 4 for each of the comparisons, along with their fold changes and adjusted P values. Various categories of genes showed differential expression: cell surface markers (HLA-DRB1, CD19, and CD24), cytokines/chemokines (IL-1B, IL-19, CXCL5, CCL5,

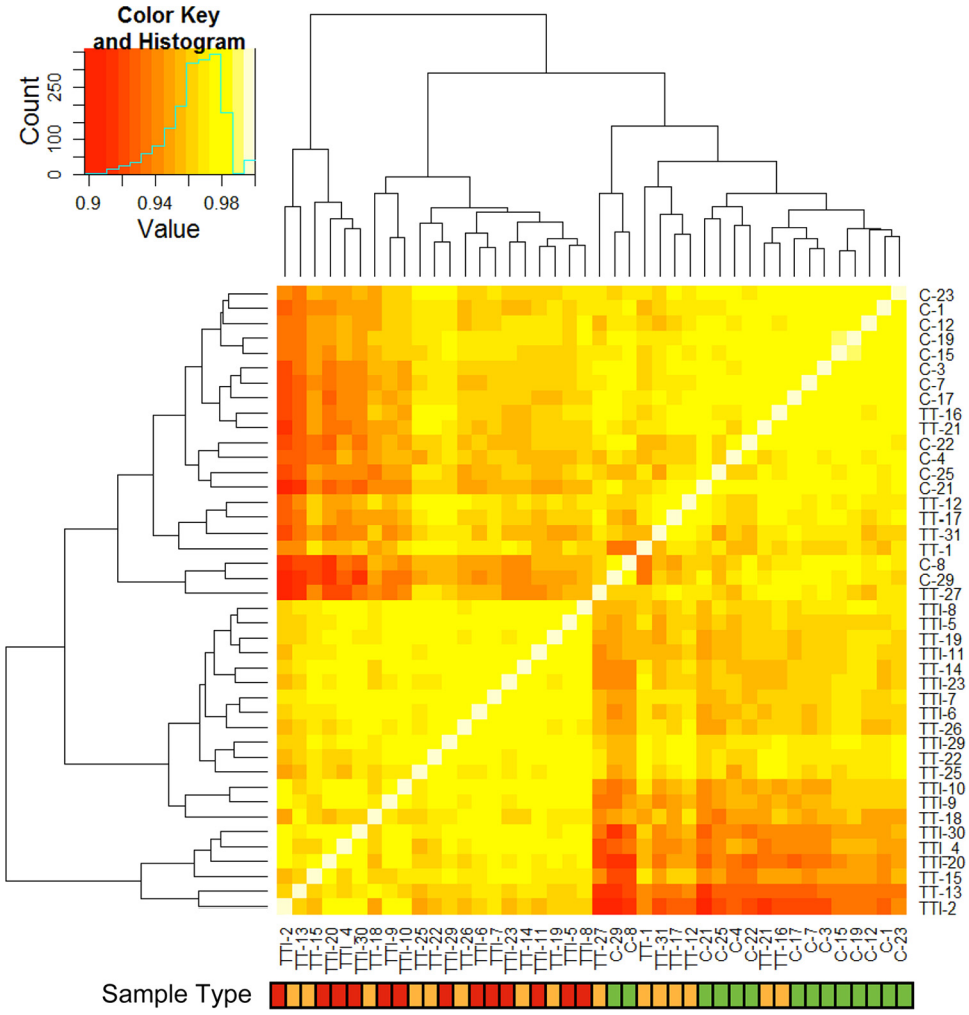

FIG. 1. Global gene expression. A correlation matrix for the global gene expression profile (all genes) between all pairs of samples. Samples are ordered into a hierarchical tree for similarity. Sample type: TTI, trichiasis with inflammation (red); TT, trichiasis without inflammation (orange); C, control (green).

and CCL20), immune response effector molecules (S100A7, DEFB4, PLUNC, NOS2A, DUOX2, and INDO), extracellular matrix regulators (MMP7, MMP9, MMP12, SPARCL, and CEACAM5), and keratinization components (SPRR2A, SPRR2D, KRT6A, and KRT6B). Factors involved in the innate immune response were particularly prominent (S100A7, CXCL5, DEFB4, PLUNC, NOS2A, and SAA1). There was some evidence of activated T_H1 responses in cases (INDO, DUOX2, and NOS2A). There was no evidence of increased abundance of transcripts associated with a T_H2 type response

TABLE 3. Number of regulated genes in each of three comparisons, filtered by different adjusted *P* value thresholds[a]

P value threshold	TTI vs C		TT vs C		TTI vs TT	
	No. of genes	%	No. of genes	%	No. of genes	%
Adjusted						
<0.05	9,003	52	4,141	24	4,677	27
<0.01	6,766	38	2,041	12	1,644	9
<0.001	4,423	25	841	5	189	1
Filtered[b]						
Upregulated genes	2,022		410		128	
Downregulated genes	2,401		431		61	

[a] The comparisons included groups TTI versus C, TT versus C, and TTI versus TT. The percent values are of the total number of genes selected for normalization and analysis (n = 17,214).

[b] That is, filtered at the adjusted *P* value of <0.001.

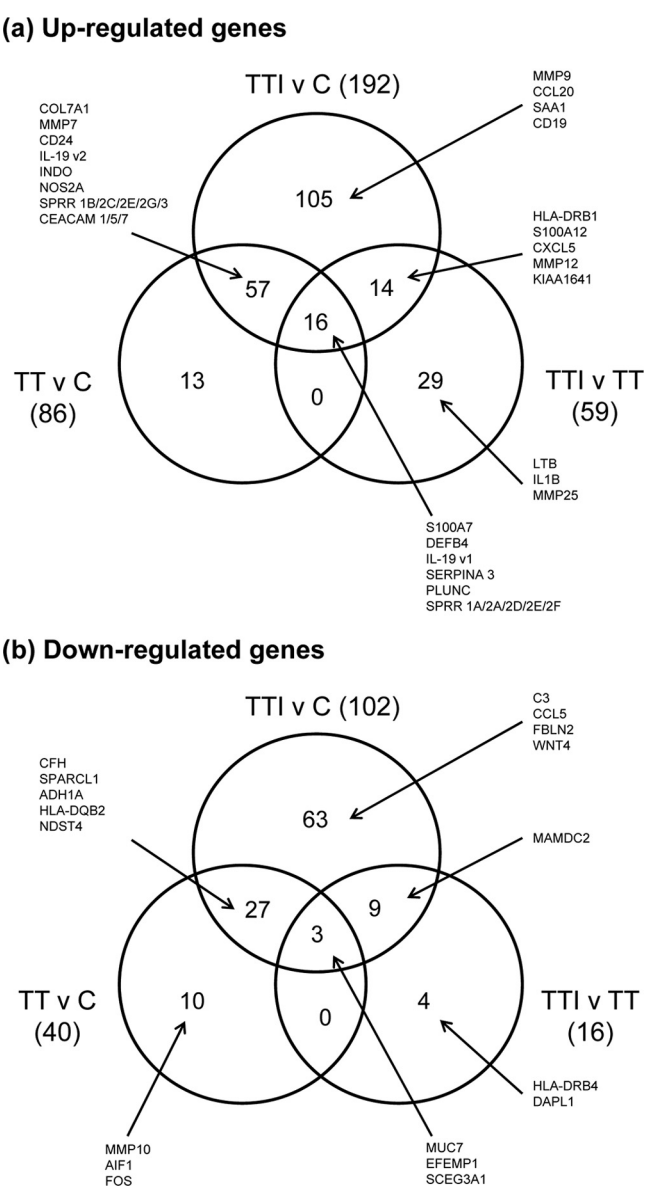

FIG. 2. Differentially expressed genes with a >2-fold change for the three comparison pairs (TTI versus C, TT versus C, and TTI versus TT). (a) Upregulated genes; (b) downregulated genes. Selected genes of interest are shown.

in cases. Table S1 in the supplemental material presents the fold changes and adjusted *P* values for all targets where there was a >2-fold increase or decrease.

Gene enrichment analysis. The results of gene enrichment analysis performed using DAVID and GeneGO are shown in Table 5. In comparing samples from trichiasis cases (with or without inflammation), we found that DAVID identified significant enrichment of gene ontology (GO) terms involved in the development of a keratinized/cornified squamous epithelial surface in individuals with trichiasis (irrespective of the presence of inflammation). The top five GO terms for the comparisons of the TT or TTI groups with the C group reflect these processes, although there is considerable overlap in the specific genes included in these terms. Where clinical inflammation was also present, additional GO terms related to immune responses were also enriched. Pathway analysis using GeneGO identified a similar pattern of changes with trichiasis associated with cytoskeleton remodeling (keratin, WNT pathway) and regulation of cell cycle components and inflamed conjunctiva associated with an immunological response.

Quantitative RT-PCR analysis of gene expression. Quantitative real-time RT-PCR was performed for the 16 gene expression targets (Table 6) on all 772 cases and controls. The geometric means, the 95% confidence interval (95% CI) values, and statistical comparisons of the three clinical categories are shown in Table 6. The fold changes measured by the microarray experiment were highly correlated with those measured by quantitative RT-PCR: correlation coefficient 0.88, $P < 0.0001$. A high proportion (42%) of samples did not have detectable expression of *IL19*. However, there was a significant difference (odds ratio, 9.4; 95% CI, 6.7 to 13.1; $P < 0.001$) in the proportion expressing *IL19* between the TT/TTI cases (319/386 [83%]) and controls (130/386 [34%]]).

There was no evidence that *IFN*γ was differentially regulated between cases and controls; however, there was significantly increased expression of both *INDO* and *NOS2A*. The expression of *IL13* was lower in cases compared to controls, particularly where inflammation was also present. Conversely, the expression of the decoy receptor *IL13RA2* was increased in the cases compared to the controls. The ratio of *IL13*/*IL13RA2* expression was markedly reduced in cases relative to controls. We found the expression of *ARG1* to also be reduced in cases relative to controls.

IL1B, *CXCL5*, and *S100A7* were expressed at significantly increased levels in cases compared to controls. Cases with inflammation (TTI) were found to have increased expression of these three factors relative to cases without inflammation; however, the critical significance level of $P < 0.001$ was not reached. We did not find a variation in the expression of *IL10* between cases and controls. The extracellular matrix regulators *MMP7*, *MMP9*, *MMP12*, and *HAS3* were increased in cases relative to controls.

We examined whether there was any evidence of differential gene expression between controls with mild conjunctival scarring and controls without any sign of scarring. The only difference was for *NOS2A*, which was slightly increased in the controls with mild scarring (fold change, 1.54; $P < 0.0001$).

***C. trachomatis* PCR.** All 772 dry samples collected from individuals in the case-control study were tested for *C. trachomatis* by PCR. Of these, only one sample was positive, five were consistently inhibited on retesting, and the remaining 766 tested negative. All positive and negative controls performed normally. With the exception of the five inhibited samples, all specimen internal control tests were positive.

DISCUSSION

The pathophysiology of scarring trachoma is poorly characterized in terms of the immunological factors and fibrogenic pathways involved. In the present study, using transcriptome-wide gene expression analysis, we found marked differential gene expression in the conjunctiva of individuals with significant trachomatous scarring and trichiasis compared to controls with minimal or no conjunctival scarring. Gene enrichment

TABLE 4. Genes of interest with the largest fold changes, as determined by three pairwise comparisons[a]

Gene	Description	Function	TTI vs C		TT vs C		TTI vs TT	
			FC	*P*	FC	*P*	FC	*P*
Cell surface markers/receptor								
HLA-DRB1	Major histocompatibility complex DRB1	MHC class II molecule	4.0	0.008	0.43	0.809	4.7	0.009
HLA-DQB2	Major histocompatibility complex DQB2	MHC class II molecule	0.29	2×10^{-5}	0.50	0.021	0.57	0.056
CD19		B-cell surface	2.2	4×10^{-4}	1.2	0.535	1.9	0.011
CD24		B-cell and granulocyte surface marker/cell adhesion	3.5	4×10^{-7}	1.6	7×10^{-4}	1.2	0.540
Cytokines/chemokines								
IL1B	IL-1β	Proinflammatory cytokine; released by epithelium and other cells	1.9	0.076	0.59	0.189	3.2	0.005
IL10	IL-10	Anti-inflammatory cytokine	1.6	3×10^{-4}	1.2	0.288	1.4	0.027
IL19	IL-19	Uncertain, possibly proinflammatory	13.4	3×10^{-7}	4.2	0.003	1.8	0.016
IL23A	IL-23	Proinflammatory cytokine; promotes IL-17 response	1.6	4×10^{-4}	1.2	0.098	1.2	0.103
CXCL5	Chemokine (CXC) ligand 5	Chemotactic neutrophil	5.3	1×10^{-6}	1.1	0.790	4.8	1×10^{-4}
CCL5	Chemokine (CC) ligand 5/RANTES	Chemotactic for lymphocytes, eosinophils, basophils	0.22	5×10^{-6}	0.29	8×10^{-4}	1.1	0.334
CCL20	Chemokine ligand 20	Macrophage inflammatory protein 3, lymphocyte chemotaxis	2.4	0.003	1.3	0.437	1.8	0.060
Immune response effector molecules								
S100A7	Psoriasin 1	Antimicrobial, chemokine, cornified envelope precursor	37.1	7×10^{-9}	9.6	6×10^{-5}	3.8	0.014
S100A12	EN-RAGE	Proinflammatory protein, secreted by neutrophils	3.3	6×10^{-5}	1.5	0.191	2.2	0.013
DEFB4	Defensin β4	Mucosal innate immunity, antibacterial, chemotactic	11.3	2×10^{-6}	3.8	0.008	2.9	0.029
DEFB103A	Defensin β103A/defensin β3	Mucosal innate immunity, antibacterial, chemotactic	3.1	0.001	2.0	0.076	1.6	0.225
PLUNC	Palate, lung, nasal epithelium associated	Mucosal defense, antibacterial, antiviral, from epithelium	7.4	2×10^{-8}	2.7	0.007	2.8	0.006
NOS2A	Nitric oxide synthase 2A	Generates nitric oxide, antichlamydial	3.5	3×10^{-6}	1.8	0.005	1.3	0.336
DUOX2	Dual oxidase 2	Produce ROS in response to T_H1 stimulation	3.4	5×10^{-8}	2.3	9×10^{-5}	1.5	0.0515
GPX2	Glutathione peroxidase 2	H_2O_2 reducing activity in epithelium	5.0	5×10^{-10}	3.1	2×10^{-6}	1.6	0.021
INDO	Indoleamine-pyrrole 2,3-dioxygenase	Catalyzes tryptophan (antichlamydial), immunoregulatory	4.1	3×10^{-6}	3.0	4×10^{-4}	1.4	0.322
SAA1	Serum amyloid A1	Promotes inflammation, promotes collagenase activity	3.9	1×10^{-4}	1.5	0.071	1.7	0.066
SERPINA3	α1-Antitrypsin	Acute-phase protein, inhibits proteases (MMP9)	9.7	4×10^{-10}	2.4	0.003	4.0	1×10^{-4}
CFH	Complement factor H	Regulates the alternative complement pathway	0.30	2×10^{-7}	0.36	3×10^{-6}	0.71	0.119
Extracellular matrix regulators, components, and adhesion								
MMP1	Matrix metalloproteinase 1	MMP with multiple substrates	1.5	0.025	1.8	0.004	1.2	0.379
MMP7	Matrix metalloproteinase 7	Degrades collagen I/IV, laminin, and fibronectin; activates defensins	3.5	1×10^{6}	3.6	6×10^{-6}	1.0	0.925
MMP9	Matrix metalloproteinase 9	MMP with multiple substrates: ECM components and cytokines	2.4	3×10^{-4}	1.3	0.403	1.9	0.013
MMP10	Matrix metalloproteinase 10	MMP involved in wound healing	1.6	0.045	2.9	1×10^{-4}	1.8	0.031
MMP12	Matrix metalloproteinase 12	MMP with multiple substrates	3.1	2×10^{-5}	1.0	0.833	1.4	0.003
SPARCL1	Hevin	Extracellular matrix regulator	0.14	6×10^{-7}	0.24	2×10^{-4}	0.66	0.170
COL7A1	Collagen 7α1	Epithelial basement zone	2.5	6×10^{-6}	2.0	8×10^{-4}	1.2	0.276
HAS3	Hyaluronan synthase 3	Synthesizes hyaluronic acid, an extracellular matrix component	3.6	3×10^{-9}	2.0	2×10^{-4}	1.8	0.002
ARG1	Arginase	Converts L-arginine to L-ornithine	1.1	0.288	1.2	0.205	1.0	0.766

ANKRD36B	Ankyrin repeat domain 36B	KIAA1641	5.6	4×10^{-10}	1.9	0.004	3.0	6×10^{-5}
CEACAM1	Carcinoembryonic antigen-1	Cell adhesion molecule	2.2	1×10^{-5}	2.2	5×10^{-5}	1.1	0.247
CEACAM5	Carcinoembryonic antigen-5	Cell adhesion molecule	8.6	2×10^{-10}	5.4	1×10^{-7}	1.6	0.074
CEACAM7	Carcinoembryonic antigen-7	Cell adhesion molecule	4.8	7×10^{-7}	3.4	7×10^{-5}	1.3	0.343
NDST4	*N*-Deacetylase/*N*-sulfotransferase 4	Heparin biosynthesis	0.1	1×10^{-9}	0.14	1×10^{-7}	0.71	0.251
Epithelial components								
SPRR1A	Small proline-rich protein 1A	Cornified envelope precursor	4.8	4×10^{-5}	2.4	0.029	2.0	0.074
SPRR2A	Small proline-rich protein 2A	Cornified envelope precursor	13.6	3×10^{-9}	4.8	6×10^{-5}	2.8	0.008
SPRR2D	Small proline-rich protein 2D	Cornified envelope precursor	10.5	7×10^{-8}	3.7	0.002	3.0	0.009
SPRR2F	Small proline-rich protein 2F	Cornified envelope precursor	11.8	8×10^{-9}	4.4	1×10^{-4}	2.7	0.009
KRT6A	Keratin 6A	Keratin	10.3	1×10^{-8}	5.5	1×10^{-5}	1.9	0.068
KRT6B	Keratin 6B	Keratin	5.0	5×10^{-8}	2.7	4×10^{-4}	1.9	0.019
KRT6E	Keratin 6E	Keratin	2.8	0.020	3.4	0.014	0.43	0.733
KRT17	Keratin 17	Keratin	3.6	3×10^{-5}	1.6	0.168	2.3	0.009
KRT24	Keratin 24	Keratin	3.9	2×10^{-6}	1.6	0.099	2.5	0.002
MUC1	Mucin 1	Epithelial cell-derived, surface-bound mucin	2.2	2×10^{-8}	1.7	6×10^{-5}	1.3	0.037
MUC4	Mucin 4	Epithelial cell-derived, surface-bound mucin	3.2	2×10^{-7}	1.8	0.005	1.8	0.008
MUC5A	Mucin 5A	Goblet cell-derived, soluble mucin	1.5	0.050	1.8	0.017	0.86	0.531
MUC7	Mucin 7	Lacrimal gland-derived, soluble mucin in tear film	0.19	3×10^{-4}	0.48	0.141	0.40	0.053

[a] FC, fold change. *P* values represent the adjusted *P* value for the specific pairwise comparison, as determined by the Benjamini-Hochberg method (3). A positive fold change indicates increased expression in the first phenotype group in each comparison.

analysis indicates that the most prominent changes were in the nature of the epithelium, suggestive of squamous metaplasia. Second, trachomatous scarring and trichiasis, particularly in the presence of clinically visible conjunctival inflammation, was associated with various proinflammatory factors, characteristic of an activated innate immune response. Third, there was very limited evidence of an ongoing T_H1 but not T_H2 response in scarred conjunctiva. Finally, there was evidence of cytoskeletal remodeling, including enrichment of the profibrotic WNT pathway and increased expression of various regulators of the extracellular matrix. We found that the results for various targets measured by quantitative PCR in a large case-control series were consistent with the microarray data.

Squamous metaplasia of the conjunctival epithelium. An important clinical feature of cicatricial trachoma is the transformation of the conjunctival epithelium from healthy mucosa into a pathologically dry, keratinized surface. Normally, the tarsal conjunctival surface is formed of a nonkeratinized stratified epithelium. The superficial cells tend to be cylindrical in shape, and there are scattered goblet cells. In advanced trachomatous scarring, the epithelium is thinned and transformed into a keratinized, stratified, squamous epithelium with loss of goblet cells (1, 4). Squamous metaplasia requires the synthesis of specific proteins, including ***s***mall ***p***roline-***r***ich ***p***roteins (SPRR) and keratins that are key components of the cornified envelope. We found significantly increased expression in cases of several SPRRs (SPRR1A, SPRR2A, SPRR2D, and SPRR2F) and keratins (KRT6A, KRT6B, KRT6E, KRT17, and KRT24). This was more marked in clinically inflamed conjunctiva. Extensive squamous metaplasia is detrimental to the health of the ocular surface and may contribute to the development of blinding corneal opacification in trachoma.

Ocular surface squamous metaplasia develops in other conjunctival disease processes, often in association with chronic inflammation and dryness, as in, for example, ocular cicatricial pemphigoid, Stevens-Johnson syndrome, systemic sclerosis, and alkali burns. It is likely that squamous metaplasia is a nonspecific response to injury or inflammation. In experimental models of dry eye disease there is an influx of inflammatory cells, particularly $CD4^+$ lymphocytes, and upregulation of SPRRs (SPRR-1, SPRR-2) under the influence of proinflammatory cytokines, particularly IL-1β and gamma interferon (IFN-γ) (17, 39).

Mucins are important components of the ocular surface. Their hydrophilic properties allow surface wetting, and they provide a physical barrier to bacterial invasion (42). We found some changes in the expression of several mucins in TT cases. There was increased expression of *MUC1* and *MUC4*, which are produced by epithelial cells and attach to the surface membrane. In addition, there was a nonsignificant increase in the expression of *MUC5A*, which is a large soluble protein released from goblet cells. *MUC7* expression was reduced, although the significance of this is unclear since this is primarily derived from the lacrimal gland. Increased expression of these mucins may be to compensate for a dry ocular surface.

Proinflammatory response. Chronic conjunctival inflammation is considered to be a key event in the pathophysiology of trachomatous scarring. Children with active trachoma, particularly severe inflammatory trachoma (TI), are at increased risk of developing the scarring complications of this disease in later

TABLE 5. Results of gene set enrichment analysis[a]

Comparison	Gene Ontology terms (DAVID)				Gene-GO	
	N	Name	FC	*P*	Pathway	*P*
TTI vs C	104	GO:0007398~ectoderm development	4.04	1.28×10^{-09}	Cytoskeleton remodeling–keratin filaments	9.60×10^{-12}
		GO:0008544~epidermis development	4.06	5.14×10^{-09}	DNA damage–ATM/ATR regulation of G_1/S checkpoint	1.41×10^{-10}
		GO:0031424~keratinization	8.03	1.67×10^{-07}	Cell cycle–the metaphase checkpoint	8.65×10^{-10}
		GO:0030216~keratinocyte differentiation	6.09	3.46×10^{-07}	Cytoskeleton remodeling–cytoskeleton remodeling	2.04×10^{-9}
		GO:0009913~epidermal cell differentiation	5.58	9.93×10^{-07}	Apoptosis and survival–TNFR1 signaling pathway	5.47×10^{-9}
		GO:0006955~immune response	2.08	1.45×10^{-06}	DNA damage–NHEJ mechanisms of DSB repair	9.72×10^{-9}
		GO:0030855~epithelial cell differentiation	3.77	5.29×10^{-06}	Cell cycle–cell cycle (generic schema)	1.02×10^{-8}
		GO:0006952~defense response	2.01	2.17×10^{-05}	Apoptosis and survival–FAS signaling cascades	3.27×10^{-8}
		GO:0009611~response to wounding	2.01	9.39×10^{-05}	Cell cycle–role of SCF complex in cell cycle regulation	5.31×10^{-8}
		GO:0009617~response to bacterium	2.83	1.37×10^{-04}	Cytoskeleton remodeling–TGF, WNT, and cytoskeletal remodeling	6.41×10^{-8}
TT vs C	37	GO:0007398~ectoderm development	10.68	1.89×10^{-27}	Signal transduction–AKT signaling	7.97×10^{-9}
		GO:0008544~epidermis development	10.91	2.65×10^{-26}	Development–PIP3 signaling in cardiac myocytes	5.18×10^{-8}
		GO:0030216~keratinocyte differentiation	18.64	2.87×10^{-21}	Cell cycle–role of Nek in cell cycle regulation	5.37×10^{-7}
		GO:0009913~epidermal cell differentiation	17.04	2.43×10^{-20}	Transport–RAN regulation pathway	5.82×10^{-7}
		GO:0030855~epithelial cell differentiation	10.85	1.40×10^{-19}	Cell cycle–regulation of G_1/S transition (part 1)	1.35×10^{-6}
		GO:0031424~keratinization	22.48	6.79×10^{-18}	Cytoskeleton remodeling–keratin filaments	3.40×10^{-6}
		GO:0060429~epithelium development	6.54	5.43×10^{-14}	DNA damage–ATM/ATR regulation of G_1/S checkpoint	3.61×10^{-6}
		GO:0018149~peptide cross-linking	12.51	9.62×10^{-05}	Cell cycle–nucleocytoplasmic transport of CDK/cyclins	3.61×10^{-6}
		GO:0007586~digestion	5.36	2.64×10^{-04}	Cell cycle–spindle assembly and chromosome separation	5.61×10^{-6}
		GO:0009617~response to bacterium	3.56	5.66×10^{-04}	Development–IGF-1 receptor signaling	6.18×10^{-6}
TTI vs TT	105	GO:0006955~immune response	5.07	1.69×10^{-24}	Cell adhesion–chemokines and adhesion	1.80×10^{-9}
		GO:0006952~defense response	4.65	2.36×10^{-19}	Immune response–CCR3 signaling in eosinophils	4.79×10^{-9}
		GO:0042330~taxis	8.31	4.23×10^{-14}	Immune response–immunological synapse formation	6.28×10^{-8}
		GO:0006935~chemotaxis	8.31	4.23×10^{-14}	Cytoskeleton remodeling–cytoskeleton remodeling	1.67×10^{-7}
		GO:0009611~response to wounding	3.83	2.74×10^{-11}	Apoptosis and survival–BAD phosphorylation	2.01×10^{-7}
		GO:0007626~locomotory behavior	5.28	5.57×10^{-11}	Development–IGF-1 receptor signaling	3.29×10^{-7}
		GO:0006954~inflammatory response	4.64	3.45×10^{-10}	Immune response–IL-10 signaling pathway	5.74×10^{-7}
		GO:0007610~behavior	3.57	9.79×10^{-09}	Development–thrombopoietin-regulated cell processes	7.52×10^{-7}
		GO:0009617~response to bacterium	5.30	1.28×10^{-07}	Immune response–IL-4–antiapoptotic action	7.93×10^{-7}
		GO:0007398~ectoderm development	4.73	1.44×10^{-06}	Chemotaxis–leukocyte chemotaxis	1.02×10^{-6}

[a] The top-ten gene enrichment terms were identified by using (i) the Database for Annotation, Visualization, and Integrated Discovery (DAVID), v6.7, and (ii) Gene-GO. FC, fold change. *N*, number of gene ontology (GO) terms where $P < 0.05$ and fold enrichment was >1.5.

TABLE 6. Geometric means and 95% confidence intervals for the expression of each gene by clinical category[a]

Gene	C (n = 386)		TT (n = 142)		TTI (n = 244)		TTI vs C		TT vs C		TTI vs TT		P^d
	Mean	95% CI	Mean	95% CI	Mean	95% CI	FC	P^b	FC	P^b	FC	P^c	
IFNγ	0.0212	0.0191–0.0235	0.0194	0.0167–0.0225	0.0178	0.0157–0.0203	0.78	0.0433	1.03	0.8282	0.92	0.4293	0.1105
TNFα	0.2064	0.1964–0.2169	0.2261	0.2013–0.2539	0.2370	0.2150–0.2613	1.11	0.0767	1.16	0.0403	1.05	0.5474	0.0236
IL1B	0.6248	0.5763–0.6775	1.1038	0.9131–1.3343	1.4000	1.2127–1.6164	2.18	<0.0001	1.86	<0.0001	1.27	0.0489	<0.0001
IL10	0.0361	0.0321–0.0407	0.0429	0.0346–0.0532	0.0418	0.0358–0.0487	1.08	0.4941	1.34	0.0660	0.97	0.8449	0.2055
IL13	0.0018	0.0016–0.0022	0.0012	0.0007–0.0015	0.0009	0.0007–0.0011	0.45	0.0002	0.72	0.0545	0.75	0.3895	<0.0001
IL13RA2	0.0083	0.0075–0.0093	0.0184	0.0153–0.0223	0.0178	0.0149–0.0214	2.17	<0.0001	2.12	<0.0001	0.97	0.7785	<0.0001
IL19[e]	0.00003	0.00002–0.00004	0.0001	0.00007–0.00014	0.0001	0.00009–0.00016	2.98	0.0001	3.10	0.0227	1.29	0.2400	<0.0001
ARG1	0.0331	0.0310–0.0353	0.0408	0.0349–0.0476	0.0432	0.0383–0.0489	1.30	0.0007	1.24	0.0109	1.06	0.5599	0.0001
NOS2A	0.7908	0.7247–0.8629	2.0494	1.7665–2.3776	2.4573	2.1770–2.7737	3.28	<0.0001	2.36	<0.0001	1.20	0.0671	<0.0001
INDO	1.0955	0.9907–1.2113	3.4223	2.9046–4.0323	4.3953	3.8411–5.0295	4.10	<0.0001	3.02	<0.0001	1.28	0.0232	<0.0001
CXCL5	0.0070	0.0058–0.0084	0.0248	0.0178–0.0344	0.0419	0.0323–0.0543	6.39	<0.0001	3.15	<0.0001	1.69	0.0145	<0.0001
S100A7	0.3972	0.3568–0.4421	3.4075	2.4726–4.6959	5.8468	4.5231–7.5579	14.61	<0.0001	8.69	<0.0001	1.72	0.0109	<0.0001
MMP7	1.0005	0.9169–1.0917	2.7078	2.2781–3.2186	3.1925	2.8415–3.5868	3.14	<0.0001	2.79	<0.0001	1.18	0.1079	<0.0001
MMP9	0.0340	0.0305–0.0378	0.0570	0.0478–0.0679	0.0633	0.0554–0.0723	1.88	<0.0001	1.65	0.0001	1.11	0.3429	<0.0001
MMP12	0.4047	0.3662–0.4473	0.7397	0.6247–0.8759	0.8437	0.7329–0.9714	2.08	<0.0001	1.84	<0.0001	1.14	0.2501	<0.0001
HAS3	0.1456	0.1313–0.1613	0.2232	0.1904–0.2619	0.2441	0.2133–0.2793	1.68	<0.0001	1.53	0.0001	1.09	0.4136	<0.0001
IL13/IL13RA2[f]	0.1911	0.1556–0.2348	0.0505	0.0340–0.0750	0.0425	0.0330–0.0547	0.20	<0.0001	0.28	0.0001	0.84	0.4435	<0.0001

[a] The expression levels are standardized relative to the expression of *HPRT1* in the same reaction. FC, fold change (a value of >1 indicates increased expression in the first clinical category of each comparison compared to the second).
[b] P values for paired t test. Using the Bonferroni correction, the critical significance threshold level is a P value of <0.001.
[c] P values for unpaired t test. Using the Bonferroni correction, the critical significance threshold level is a P value of <0.001.
[d] As determined by analysis of variance of the expression level of the gene across the three clinical categories.
[e] For *IL19*, only cases or controls where expression was detected are included.
[f] That is, the ratio of *IL13*/*IL13RA* expression.

life (16, 71). In adults with scarring the conjunctiva frequently appears clinically inflamed, although in many settings *C. trachomatis* infection is only infrequently detected, as was the case in our study (12, 62, 72). Individuals with TT or conjunctival scarring (TS) are at increased risk of nonchlamydial bacterial infection, which has been associated with clinically visible conjunctival inflammation.(8, 12).

In the present study we found increased expression of proinflammatory mediators in cases (*S100A7*, *S100A12*, *CXCL5*, *CCL20*, *IL1B*, and *SAA1*). Interestingly, these factors were significantly raised in cases without clinical inflammation compared to controls, albeit to a lesser extent than in the inflamed cases. Several factors were characteristic of innate immune responses. *In vitro* studies of epithelial cells infected with *C. trachomatis* have measured the production of various chemokines and cytokines (52, 56). These experiments have consistently found that *C. trachomatis* infection triggers the prolonged production of a range of proinflammatory mediators, which are probably very important in the initial innate immune response to infection (IL-1α, IL-1β, IL-6, IL-8, CXCL1, CXCL2, CXCL5, and CXCL6). It is likely that some of these mediators are produced by the conjunctival epithelium *in vivo*, suggesting that the epithelium is not just an injured bystander in *C. trachomatis* infection but is actively involved in the immunofibrogenic response (56). It is also likely that nonchlamydial bacterial infections stimulate the inflammatory response, especially where there is established tissue disruption caused by repeated *C. trachomatis* infection.(10) In addition to the epithelium, other cells, such as macrophages and neutrophils, probably contribute to the production of these proinflammatory mediators.

The innate immune response of epithelial cells is triggered through the stimulation of pattern recognition receptors (PRRs) by bacterial antigens. These receptors recognize conserved pathogen-associated molecular patterns (PAMPs). PRRs, such as the Toll-like receptors (TLRs), detect PAMPs both on the epithelial cell surface and in the intracellular compartment. Relatively little is known about the role of PRRs in human *C. trachomatis* infection in general, and there are virtually no data for trachoma. *C. trachomatis* recognition by PRRs TLR2 and TLR4 is the most well-characterized pathway, with *in vitro* studies indicating that both can detect a range of *C. trachomatis* antigens (2, 35, 48). Their specificity appears to differ; TLR4 detects *C. trachomatis* lipopolysaccharide (LPS) and HSP60, while TLR2 appears to detect a ligand produced by viable *C. trachomatis* (6). In women, *C. trachomatis*-induced tubal scarring has recently been associated with genetic variation in TLR2 defined by SNP haplotypes (37). In a mouse model of *C. trachomatis* genital tract infection, recognition of *C. trachomatis* through TLR2 was associated with increased production of proinflammatory cytokines (IL-1, IL-6, MIP-2, and TNF-α) and the development of scarring in the oviduct (14). TLR2-knockout mice developed much less scarring, although the duration of infection was not affected.

We found increased expression of *IL1B* in cases of trichiasis compared to controls, irrespective of clinical inflammation. The role of IL-1β has previously been investigated in trachoma. Immunohistochemistry of biopsy tissue from children with active trachoma localized the production of IL-1α and IL-1β to the superficial layers of epithelial cells, with some additional production by stromal macrophages (22). We have previously reported increased *IL1B* expression in both active trachoma and conjunctival scarring (9, 10). In both of these clinical groups, increased *IL1B* expression occurred with or without *C. trachomatis* infection. In individuals with established conjunctival scarring, nonchlamydial bacterial infection was associated with increased expression of *IL1B*, suggesting that such infections can promote a proinflammatory response (10). Others have also found in a separate population that tear fluid from people with TTI has increased IL-1β compared to

controls (59). There is a growing body of evidence which indicates that *C. trachomatis* and other nonchlamydial bacterial infections stimulate the production of IL-1β through a combination of PRRs, leading to a cascade of proinflammatory products which both help to combat infection but at the same time can damage tissues (50, 65). In a number of different scarring disease processes, IL-1β has been identified as an important cytokine in fibrogenesis, possibly through the activation of fibroblasts either via platelet-derived growth factor or transforming growth factor β (TGF-β; activated via MMP9) (24, 58).

In the present study, CXCL5 was the chemokine with the greatest increase in expression. CXCL5 induces neutrophil chemotaxis and activation; it has also been found to induce fibroblast precursor chemotaxis and differentiation (46). Epithelial cells are probably the main source, although both macrophages and fibroblasts also produce it. A study of a *C. trachomatis*-infected epithelial cell line found CXCL5 was produced in abundance and led to neutrophil chemotaxis (74). Interestingly, there is some evidence that *C. trachomatis* antigens alone (such as MOMP or LPS) might be sufficient to stimulate CXCL5 production (74). In our study, CXCL5 expression was increased in the absence of detectable *C. trachomatis*, raising the question of what might be stimulating its production. Models of *Escherichia coli* LPS-induced pulmonary inflammation indicate that CXCL5 is produced by the epithelium through TLR4-dependent signaling (34). It is therefore possible that in the context of cicatricial trachoma other bacterial pathogens could stimulate CXCL5 production directly or indirectly through other cytokines (IL-1β and TNF-α) or products of extracellular matrix (ECM) breakdown (actin) (67).

S100A7 (Psoriasin 1) was the most strongly upregulated gene in the present study. Initially identified in psoriatic skin lesions, it is a member of the S100 family, a diverse group of calcium-binding proteins involved in various processes, including cell proliferation and differentiation. It is produced by keratinocytes and has a proinflammatory, microbicidal action, promoting production of IL-8, TNF-α, CCL3, and CCL20 (75). S100A7 has been implicated in several disease processes. It is highly expressed in ductal carcinoma *in situ*, where it is increased by proinflammatory cytokines (IL-1, IL-6, IL-17, and TNF-α) (70). Similarly, in models of skin inflammation, S100A7 is produced in response to these cytokines (26). S100A7 is proposed as a novel biomarker for Alzheimer's disease; its concentration in the cerebrospinal fluid and expression in the brain are increased with increasing severity of disease (51). It has an important antibacterial action in skin both directly through a porin-like action on bacterial cell walls and indirectly through stimulation of the innate immune response. S100A7 induces neutrophil chemotaxis to sites of infection and at higher concentration stimulates them to degranulate, produce reactive oxygen species, and release proinflammatory cytokines (IL-6, IL-8, TNF-α, CCL3, CCL4, and CCL20) (75).

The effector cells of the innate immune system (neutrophils, macrophages, and natural killer cells) are found in abundance in the conjunctiva of children with active trachomatous inflammation and in adults with trachomatous scarring and inflammation (22). It appears likely that, in established trachomatous scarring, neutrophil chemotaxis is driven by CXCL5, S100A7, and IL-1β. Animal models of *C. trachomatis* infection indicate that the greater the neutrophil infiltrate found in acute inflammation the more likely scarring complications are to develop (13, 54). Neutrophils respond to infection in a nonspecific manner, often damaging uninfected tissue. They release a range of proinflammatory chemokines/cytokines, augmenting the inflammatory response, produce reactive oxygen species and degranulate releasing a variety of molecules, such as MMP9, which can cause tissue damage. If this inflammatory response is sustained, extensive tissue damage is followed by aberrant epithelial repair in the form of scar tissue.

The microarray experiments identified several other markers suggestive of epithelial cell involvement in an innate immune response, including defensin β4 and PLUNC. These are typically nonspecific responses to bacterial infection. Serum amyloid A1 (SAA1) is an acute-phase protein, deposited at sites of inflammation. Amyloid deposition has previously been demonstrated in conjunctival biopsy tissue from individuals with trachomatous scarring (27).

Adaptive immune responses in scarring trachoma. The resolution of *C. trachomatis* infection is thought to require an IFN-γ-dependent T_H1 cell-mediated immune response. A range of data from animal models and human studies support this conclusion (5). Nude mice cannot control *C. trachomatis* genital tract infection (55); however, this function can be restored by adoptive transfer of *C. trachomatis*-specific $CD4^+$ or $CD8^+$ lymphocytes (53). Murine studies indicate the importance of IFN-γ in this response (43). We have previously reported increased *IFN*γ expression in children with active *C. trachomatis* infection, but not in those with signs of active disease in the absence of *C. trachomatis* infection (9). IFN-γ is thought to mediate its action through various mechanisms, including the production of nitric oxide free radicals by iNOS (*NOS2A*) and intracellular tryptophan depletion through the action of IDO (*INDO*). In our study of conjunctival scarring, we did not find differences in the expression of *IFN*γ between cases and controls by quantitative RT-PCR. However, there was some limited indirect evidence of a T_H1-type response associated with scarring, in that there was increased expression of *INDO* and *NOS2A*. In addition, *DUOX2* was also elevated in cases; this enzyme is found on epithelial cell surfaces and produces reactive oxygen species in response to T_H1 stimulation.

There is more uncertainty about the role of T_H2-type responses in chlamydial infection and trachoma. Earlier studies in populations where trachoma is endemic found that people with established scarring (compared to unscarred controls) had higher serum anti-*C. trachomatis* IgG titers and, when their peripheral blood mononuclear cells were stimulated with *C. trachomatis* antigens, these individuals had weaker proliferation responses and expressed IL-4 more frequently (28, 29). These observations were thought to be consistent with the hypothesis that an adverse scarred outcome is associated with a T_H2-type response. However, it was unclear whether this association simply reflected a weaker T_H1 response to infection, which was therefore more severe and/or prolonged, or whether T_H2 responses directly promoted scarring and blunted T_H1 efficacy.

Recently, there has been considerable interest in the role of IL-13 in fibrosis in a range of disease processes (73). This T_H2 cytokine is thought to promote fibrosis through several mech-

anisms including the direct stimulation of fibroblasts and the "alternative activation" of macrophages to produce arginase 1 (*ARG1*), which is important in the production of collagen. In contrast, "classically activated" macrophages produce iNOS (*NOS2A*) in response to T_H1/IFN-γ stimulation. This opposes the profibrotic action of IL-13.

We did not find any evidence of activation of T_H2 pathways in the microarray experiments. In addition, by quantitative RT-PCR, the expression of IL-13 was reduced, and the expression of *IL13RA2* (the soluble decoy receptor antagonist) was increased. The functional effect of this decreased ratio of *IL13*/*IL13RA2* is consistent with the T_H1-type inflammatory environment evident in cases.

Tissue remodeling and fibrosis. Normal tissue structure is maintained through the "dynamic equilibrium" of various cellular processes, which build up or break down the ECM. A change in the relative activity of these processes, in disease, can lead to the accumulation of scar tissue. The development of conjunctival scar tissue in trachoma is poorly understood; it is possible that several different fibrogenic pathways contribute. In the microarray analysis of gene expression in scarred conjunctiva we found evidence of increased expression of several factors that could plausibly have roles in tissue remodeling (*MMP7*, *MMP9*, *MMP12*, *HAS*, *SPARCL*, and *CEACAM* genes) and enrichment of fibrogenic pathways (TGF/WNT).

The matrix metalloproteinases are a family of complex enzymes, which are involved in a wide range of cellular processes, including the regulation of the ECM, cytokine regulation, innate immunity, and development. MMP-7 (matrilysin) had the largest fold increase in trichiasis cases. This is consistent with findings from The Gambia, where MMP-7 was found to be upregulated in TT cases (30). MMP-7 is constitutively expressed in respiratory and other epithelial cells (20). Expression is increased in damaged and bacterially infected tissue. It seems to have an important role in innate immunity, through the activation of various components (α and β defensins) and the recruitment of neutrophils.(7, 38) In addition, it regulates epithelial wound healing; MMP-7 activates MMP-9 and degrades collagen-1, collagen-4, laminin, and fibronectin. MMP-7 is an important downstream gene product of signaling in the WNT pathway.

MMP9 expression was increased in cases, particularly in the presence of inflammation. This is consistent with earlier studies in both active and scarring trachoma, in which expression levels correlated with the clinical inflammatory score (9, 10). Immunohistochemistry indicates that, in children with active trachoma, MMP-9 is found in macrophages and neutrophils (21). We have previously described an association between a coding polymorphism in *MMP9* and reduced risk of scarring (44). In a mouse model of chlamydial genital tract infection, MMP-9-knockout animals had a reduced risk of scarring sequelae (33). Together, these observations suggest a role for MMP-9 in the pathogenesis of trachoma. A major MMP-9 function is the degradation of type IV collagen present in the basement membrane that disrupts the epithelium and facilitates the migration of cells. This is thought to be an initiating event in epithelial-mesenchymal transition, a process whereby epithelial cells migrate from the surface into the stroma (36). These cells are then thought to be able to take on a fibroblast phenotype and contribute to fibrosis. It is currently unknown whether in trachoma the epithelium is a major source of fibroblasts. MMP-9 may also contribute to the development and maintenance of inflammation. Collagen fragments are both chemotactic and proinflammatory to monocytes and may linger for some time promoting a chronic inflammatory response (49).

MMP12 (macrophage metalloelastase) expression was increased to a similar extent in cases with or without inflammation. This is consistent with results from a murine model of chlamydial genital infection, which showed a modest increase in expression (54). MMP-12 is mainly produced by macrophages. It degrades elastin and may be proinflammatory (47). It is prominent in respiratory tract disease such as emphysema, where destruction of elastin is important in the pathology. It is possible that in scarring trachoma MMP-12 is important in the degradation of the ECM and the promotion of inflammation.

The cytokine TGF-β is known to be important in many scarring diseases. However, evaluation of its role in trachoma has remained elusive, partly because of its complex posttranscriptional regulation. Previously, we found no variation in the expression of *TGFβ2* (the dominant conjunctival isoform) between individuals with active trachoma, individuals with chlamydial infection, or control subjects (9). Microarray analysis can provide some insight into the possible activity of TGF-β by examining downstream pathways. In the present study, individuals with trichiasis and inflammation demonstrated enrichment of the TGF-β/WNT pathway. We subsequently confirmed, by quantitative RT-PCR, increased expression of two downstream genes in the TGF-β/WNT pathway, *NOS2A* and *MMP-7*, which have been found in other scarring disease processes (68). These observations are consistent with trachomatous scarring being driven at least in part by TGF-β, although they do not provide direct since as other factors may also increase their expression.

Limitations. Microarray analysis of the transcriptome is a very powerful technique to simultaneously examine the transcriptional activity of the genome in specific tissues. However, describing the relative abundance of a transcript is only one level of a complex biological process. Inferences about the activity of some transcripts and target genes, such as *TGFβ*, which is central to many fibrotic processes, cannot be easily investigated solely by this approach. Although we sampled in a noninvasive manner from the surface, this biases the resulting gene expression profile in favor of cells closer to the conjunctival surface. However, our recent studies of *in vitro* confocal microscopy indicate that most of the inflammatory cell activity in individuals with scarring is superficial (31). Azithromycin mass treatment had been distributed in virtually all districts where the cases and controls lived. Although azithromycin has a mild anti-inflammatory effect, this is probably relatively short-lived, and we presume that in a community treatment campaign cases and controls were equally likely to have received treatment.

Due to the severe nature of endemic trachoma in this region of Ethiopia, it is unusual to find older adults who do not have any conjunctival scarring. In order to meet the various matching criteria for the controls, we included individuals that had mild scarring, as well as controls with no scarring. However, we found only very limited evidence of any difference in gene expression between controls without scarring and those with

mild changes. This slight narrowing of the clinical distinction between cases and controls would, if anything, tend to lead to an underestimate in the size and strength of the effects measured.

Conclusions. Our findings suggest that the epithelium is not a passive bystander in cicatricial trachoma. Several proinflammatory molecules probably arise from the epithelium (IL-1B, CXCL5, and S100A7), leading to persistent recruitment of inflammatory cells into the conjunctiva, even in the absence of clinically visible inflammation. Various factors that are plausibly involved in the production of scar tissue were also detected. The driving force behind the responses at this stage of disease is not apparent. Current or recent *C. trachomatis* infection might have been expected to be associated with the inflammatory responses. However, the prevalence of *C. trachomatis* was negligible, probably reflecting the effectiveness of recent mass antibiotic treatment campaigns. Other bacteria and environmental factors could also promote inflammation in this previously damaged tissue. Longitudinal studies are needed to investigate which etiological factors and pathways are associated with progressive scarring and whether simply controlling chlamydial infection will halt progression in people with established cicatricial disease.

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