Innate Immune Responses and Modified Extracellular Matrix Regulation Characterize Bacterial Infection and Cellular/Connective Tissue Changes in Scarring Trachoma

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Trachoma is the most common infectious cause of blindness and a major public health problem in many developing countries. It is caused by recurrent ocular infection with *Chlamydia trachomatis* in childhood, with conjunctival scarring seen later in life. The pathogenesis of trachomatous scarring, however, is poorly understood, and this study was carried out to investigate the immunofibrogenic correlates of trachomatous conjunctival scarring. A case-control study of 363 cases with conjunctival scarring and 363 control participants was conducted. Investigations included *in vivo* confocal microscopy (IVCM) assessment, quantitative real-time PCR gene expression, *C. trachomatis* detection, and nonchlamydial bacterial culture. Trachomatous scarring was found to be strongly associated with a proinflammatory, innate immune response with increased expression of psoriasin, interleukin-1β, tumor necrosis factor alpha, defensin-β4A, chemokine ligand 5, and serum amyloid A1. There was also differential expression of various modifiers of the extracellular matrix, including metalloproteinases 7, 9, 10, and 12, tissue inhibitor of matrix metalloproteinase 1, and secreted protein acidic cystein-rich-like 1. The expression of many of these genes was also significantly associated with the presence of nonchlamydial bacterial infection. These infections had a marked effect on conjunctival immune processes, including an increased inflammatory infiltrate and edema seen with IVCM. This study supports the possibility that the immunofibrogenic response in scarring trachoma is partly stimulated by nonchlamydial bacterial infection, which is characterized by the expression of innate factors.

Trachoma is a major cause of blindness, especially in developing countries. Children suffer recurrent episodes of ocular infection with *Chlamydia trachomatis*, which stimulates a follicular and papillary conjunctivitis. These children are subsequently at risk of developing conjunctival scarring, which can progress to entropion (in-turning of the eyelid) and trichiasis (eyelashes rubbing against the eyeball). Corneal opacity and blindness can result, as well as severe and persistent discomfort. Trachoma is the most common infectious cause of blindness, with at least 1.3 million people estimated to be blind from the disease, 8.2 million to have trichiasis, and 40 million to have active disease (40, 52).

The pathogenesis of trachoma, and especially of the scarring process, is poorly understood. Histological studies of conjunctival biopsy specimens from scarred individuals show a chronic inflammatory cell infiltrate, especially in the substantia propria, with large numbers of lymphocytes (1, 4, 25, 51). T cells, both CD4^{+} and CD8^{+}, tend to outnumber B cells. The conjunctival stroma is replaced by thick, mostly avascular scar tissue. Earlier studies examining lymphoproliferative responses to chlamydial antigens in trachomatous subjects were consistent with findings from animal models of infection, which suggested that a strong Th1 response with production of gamma interferon (IFN-γ) was important in clearing chlamydial infection and may be protective against the development of scarring (5, 27, 28). These studies also provided limited evidence that Th2 responses were associated with conjunctival fibrosis, which may have parallels with other infectious diseases, such as schistosomiasis (59). However, recent studies using microarray analysis of conjunctival swab samples from subjects with trachomatous trichiasis have not found evidence of Th1/Th2 polarization. Instead, proinflammatory mediators, particularly suggestive of innate immune responses and factors affecting extracellular matrix (ECM) remodeling, were prominent (14, 29). A number of studies have also provided compelling evidence supporting the importance of conjunctival inflammation in the development of scarring and blinding complications (11, 18, 43, 55, 57).

Two models have been proposed to account for the pathogenesis of *C. trachomatis*-induced scarring in the eye or genital tract: the "immunological" and the "cellular" paradigms, which are not necessarily mutually exclusive (7, 16, 53). The immunological paradigm argues that cellular immune responses, especially those involving T cells, against specific chlamydial antigens are important in causing disease. The cellular paradigm proposes that host epithelial cells act as a key innate responder cell and are central in driving tissue damage.

In this case-control study we investigated the pathophysiology of trachomatous conjunctival scarring by measuring conjunctival gene expression. We examined the hypotheses that conjunctival

scarring is associated with (i) a predominantly Th2 (interleukin-13 [IL-13] mediated) rather than Th1 response, (ii) various profibrotic mediators, such as matrix metalloproteinases, and (iii) markers of innate immunity. In addition, we investigated the relationship between the expression of these various factors and both *C. trachomatis* and other bacterial infections. Finally, we related the gene expression profile to the microscopic tissue morphology changes and inflammatory cell infiltrate observed by *in vivo* confocal microscopy (IVCM). IVCM is a relatively new technique to examine the ocular surface, which provides high-resolution images down to the cellular level (31, 32).

MATERIALS AND METHODS

Ethical approval. This study adhered to the tenets of the Declaration of Helsinki. It was approved by the ethics committees of the Tanzanian National Institute for Medical Research, the Kilimanjaro Christian Medical Centre, and the London School of Hygiene and Tropical Medicine. The study was explained to potential study subjects and written, informed consent was obtained before enrollment.

Participant recruitment. The recruitment of participants into this population-based study has been previously described (30, 32). Briefly, adults with trachomatous conjunctival scarring, but without trichiasis, were recruited from an area of trachoma endemicity in the Siha district of northern Tanzania. Control subjects without scarring from the same community, frequency matched for ethnicity, were also recruited.

Clinical examination and sample collection. All participants were examined clinically, as previously described, using the 1981 World Health Organization trachoma grading system with some modifications, including a more detailed grading for conjunctival scarring (17, 30, 32). The left conjunctiva was anesthetized with preservative-free proxymetacaine 0.5% eye drops (Minims; Chauvin Pharmaceuticals) and a swab collected for microbiological analysis from the inferior fornix. Two upper tarsal conjunctival swabs were also collected (Dacron polyester-tipped; Hardwood Products Company, Guildford, ME). The first was for RNA isolation (described later) and was placed directly into a tube containing 0.2 ml of RNA stabilizer (RNA*later*; Life Technologies, United Kingdom). The second was for *C. trachomatis* detection and put into a dry tube. Samples were kept on ice packs until frozen later the same day at −80°C.

Confocal microscopy assessment. IVCM examination of the upper tarsal conjunctiva was performed using a Heidelberg Retina Tomograph 3 (HRT3) in combination with the Rostock Corneal Module (RCM) (Heidelberg Engineering GmbH, Dossenheim, Germany) according to previously described examination and image grading protocols (31). IVCM images were graded for inflammatory features and subepithelial connective tissue organization/scarring (Fig. 1 and 2).

Quantitative RT-PCR. Twenty-three different human gene transcripts were selected for quantitation by real-time reverse transcription-PCR (RT-PCR), including the following: IFN-γ (*IFNG*); indoleamine-2,3-dioxygenase (*INDO*); tumor necrosis factor alpha (*TNFA*); interleukin-1β (*IL1B*); interleukin-10 (*IL-10*); interleukin-12β (*IL12B*); interleukin 13 (*IL-13*); interleukin-13 receptor, alpha 2 (*IL13RA2*); S100 calcium-binding protein A7 (*S100A7*); defensin, beta 4A (*DEFB4A*); chemokine (C-X-C motif) ligand 5 (*CXCL5*); serum amyloid A1 (*SAA1*); arginase, liver (*ARG1*); nitric oxide synthase 2, inducible (*NOS2*); matrix metalloproteinase 1 (*MMP1*); matrix metalloproteinase 7 (*MMP7*); matrix metalloproteinase 9 (*MMP9*); matrix metalloproteinase 10 (*MMP10*); matrix metalloproteinase 12 (*MMP12*); TIMP (tissue inhibitor of matrix metalloproteinase) matrix metalloproteinase inhibitor 1 (*TIMP1*); SPARC (secreted protein, acidic, cysteine-rich)-like 1 (hevin) (*SPARCL1*); complement factor H (*CFH*); and CD83 molecule (*CD83*).

Genes were chosen to investigate the specific hypotheses outlined above. Specifically, *IFNG*, *IL12B*, *IL-13*, and *IL13RA2* expression was used to investigate a Th1/Th2 predominance; the expression of matrix metalloproteinases, *TIMP1*, and *SPARCL1* as potentially important regulators

of extracellular matrix; and *IL1B*, *TNFA*, *S100A7*, *DEFB4A*, *CXCL5*, and *SAA1* expression as evidence of a proinflammatory and/or innate immune response. We were also interested in examining evidence of differential macrophage activity (*ARG1* and *NOS2*), mature dendritic cells (*CD83*), and complement regulation (*CFH*). The selection of these genes was informed by previous studies, including a conjunctival microarray transcriptome analysis (6, 9, 14, 15, 22, 27, 28, 42, 46, 47–49). The microarray data have been deposited in NCBI's Gene Expression Omnibus under accession numbers GSE23705 (Ethiopia) and GSE24383 (Tanzania).

Total RNA was extracted from the swab sample using an RNeasy microkit (Qiagen, Hilden, Germany) and reverse transcribed to cDNA using the QuantiTect reverse transcription kit (Qiagen) according to the manufacturer's instructions. 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. Probes and primers for multiplex assays, which included up to four separate targets (including *HPRT-1*, which was used as a reference gene), were designed and synthesized by Sigma Life Science (www.sigma.com/designmyprobe; available on request). The samples were tested in duplicate, in a total reaction mixture volume of 25 μl, which contained 2 μl of sample or standard. The thermal cycle protocol used the following conditions: 95°C for 15 min, followed by 45 to 50 cycles of 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.

Microbiology samples and analysis. *C. trachomatis* DNA was detected using a PCR-based assay (Amplicor CT/NG Test; Roche) with previously described modifications (12). Samples for culture were inoculated onto blood and chocolate agar later on the day of collection (rarely more than 6 h) and incubated at 37°C for 48 h. Culture isolates were identified by standard microbiological techniques.

Data analysis. Data were entered into Access 2007 (Microsoft) and analyzed using STATA 11.0 (StataCorp LP, TX). The transcript abundances for the genes of interest were standardized relative to that of *HPRT1* in the same reaction mixture using the $\Delta\Delta C_T$ method and were normalized by $\log_{10}$ transformation (37). Linear regression models were used to estimate the fold changes (age and sex adjusted) in the levels of gene expression in those with clinically visible scarring compared to controls, subdivided into those with no/minimal inflammation (TS [inflammation grades P0 and P1]) and those with inflammation (TSI [inflammation grades P2 and P3]), where P0 is no inflammation, P1 is minimal inflammation with individual papillae prominent, P2 is moderate inflammation in which normal vessels appear hazy, and P3 is pronounced inflammation in which normal vessels on the tarsus are hidden over more than half the surface) (17). Similar fold changes were estimated for the presence of clinically significant inflammation (P2 or P3), adjusted for the level of clinical scarring as well as age and sex. Age- and sex-adjusted fold changes in gene expression by grade of IVCM connective tissue organization/scarring and for the presence of dendritiform cells were also estimated. Correlation coefficients were estimated between the increase in gene expression ($\log_{10}$ transformed) and the IVCM inflammatory infiltrate, with the significance tested using age- and sex-adjusted linear regression models. Likelihood ratio tests were used to assess the strength of association of each factor with the gene expression fold change. *P* values for all associations are shown in Results. For reference, the *P* values for the critical significance thresholds determined by a Bonferroni correction for multiple comparisons are shown in the table footnotes. However, the choice of targets for gene expression was not selected at random, as is assumed by the Bonferroni method, but contain related groups of genes which might be expected to act in a similar manner. In addition, using such a conservative correction increases the likelihood of a type 2 error.
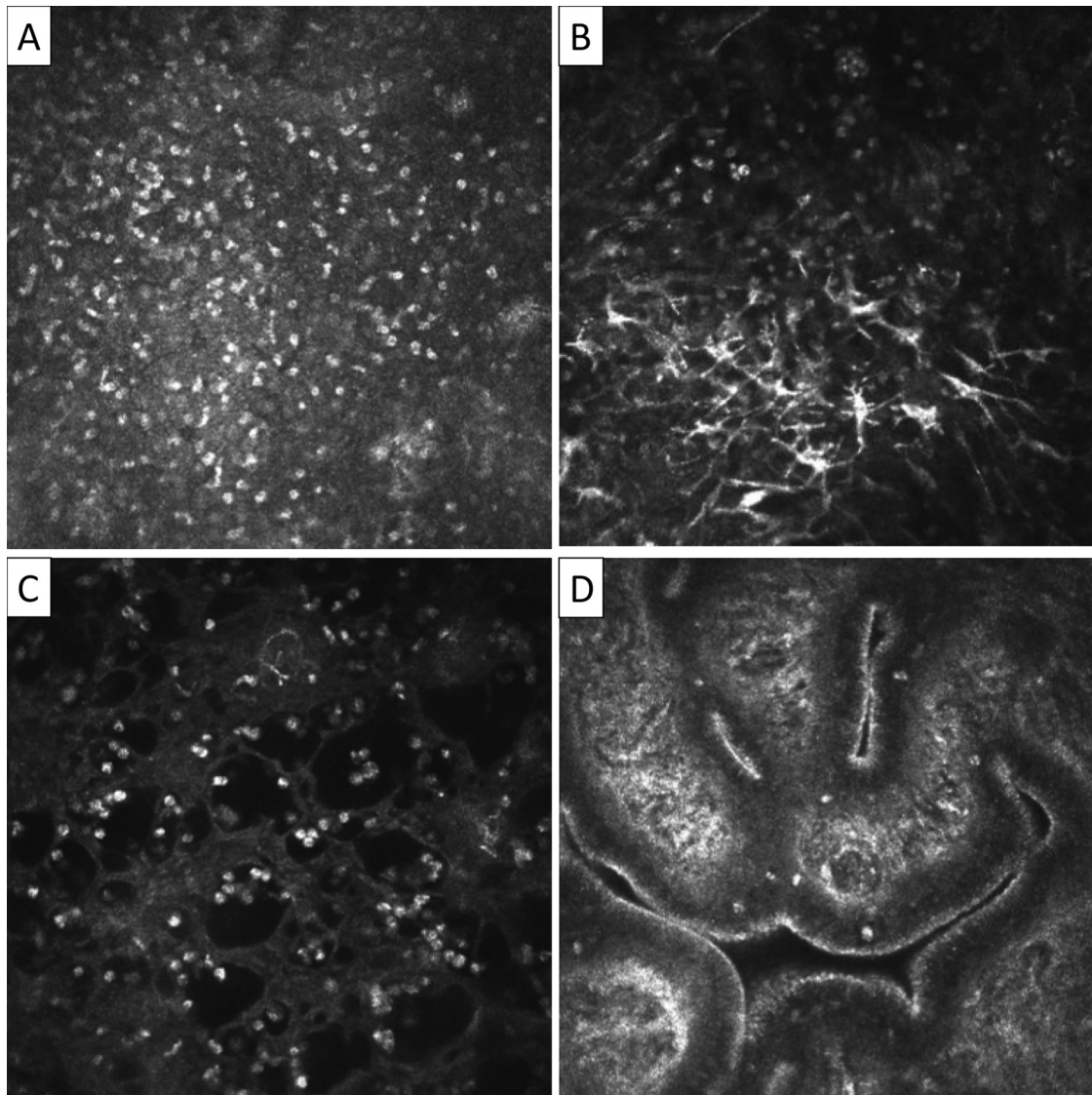
FIG 1 *In vivo* confocal microscopy grading system of inflammatory features. Images are 400 by 400 μm. (A) Inflammatory infiltrate: seen as multiple bright white nuclei. The mean inflammatory cell density of three randomly selected volume scans is calculated using the HRT/RCM software. The individual scan with the highest density of cells from within the volume scan is used for inflammatory cell density counting. (B) Dendritiform cells: graded as present or absent. To be present, the mean number of DCs per volume scan needs to be ≥1. The largest number of dendritiform cells in any individual scan in a volume scan is used for measurement. A mean number of ≥1 is used to differentiate occasional dendritiform cells seen in scans of otherwise normal subjects. (C) Tissue edema: seen as multiple black empty spaces. Graded as present or absent in any volume scan. (D) Papillae: seen as elevations with a central vascular network. Graded as present or absent in any volume scan.

RESULTS

Study participants. We recruited 363 cases with trachomatous conjunctival scarring and 363 control participants without scarring. The demographic, clinical, IVCM, and nonchlamydial bacterial infection data have previously been reported in detail (30, 32). In summary, controls were younger than the cases (mean 31.9 versus 50.3 years, $P < 0.001$), and all analyses were age and sex adjusted. Conjunctival scarring in the cases was mostly mild to moderate and cases had significantly more clinical inflammation than controls. IVCM analysis showed that the cases had more inflammatory cells and a higher connective tissue scarring score. Nonchlamydial pathogenic organisms were cultured more frequently from cases (25 [6.9%]) than from controls (7 [1.9%], $P = 0.002$). The most frequently cultured pathogenic organisms were *Haemophilus influenzae* type b and *Streptococcus pneumoniae*, while coagulase-negative staphylococci, *Corynebacterium* spp., *Streptococcus viridans*, and *Bacillus* spp. were designated as commensal organisms in this analysis. Five (1.4%) of the cases and none of the controls had *C. trachomatis* infection detected.

Conjunctival gene expression. Quantitative, real-time RT-PCR was performed for 23 gene expression targets for all 363 cases and 363 controls. There was inadequate sample for detection in four samples (three cases and one control), and amplification failed in a number of experiments for technical reasons related to laboratory power supply problems. The number of samples suc-
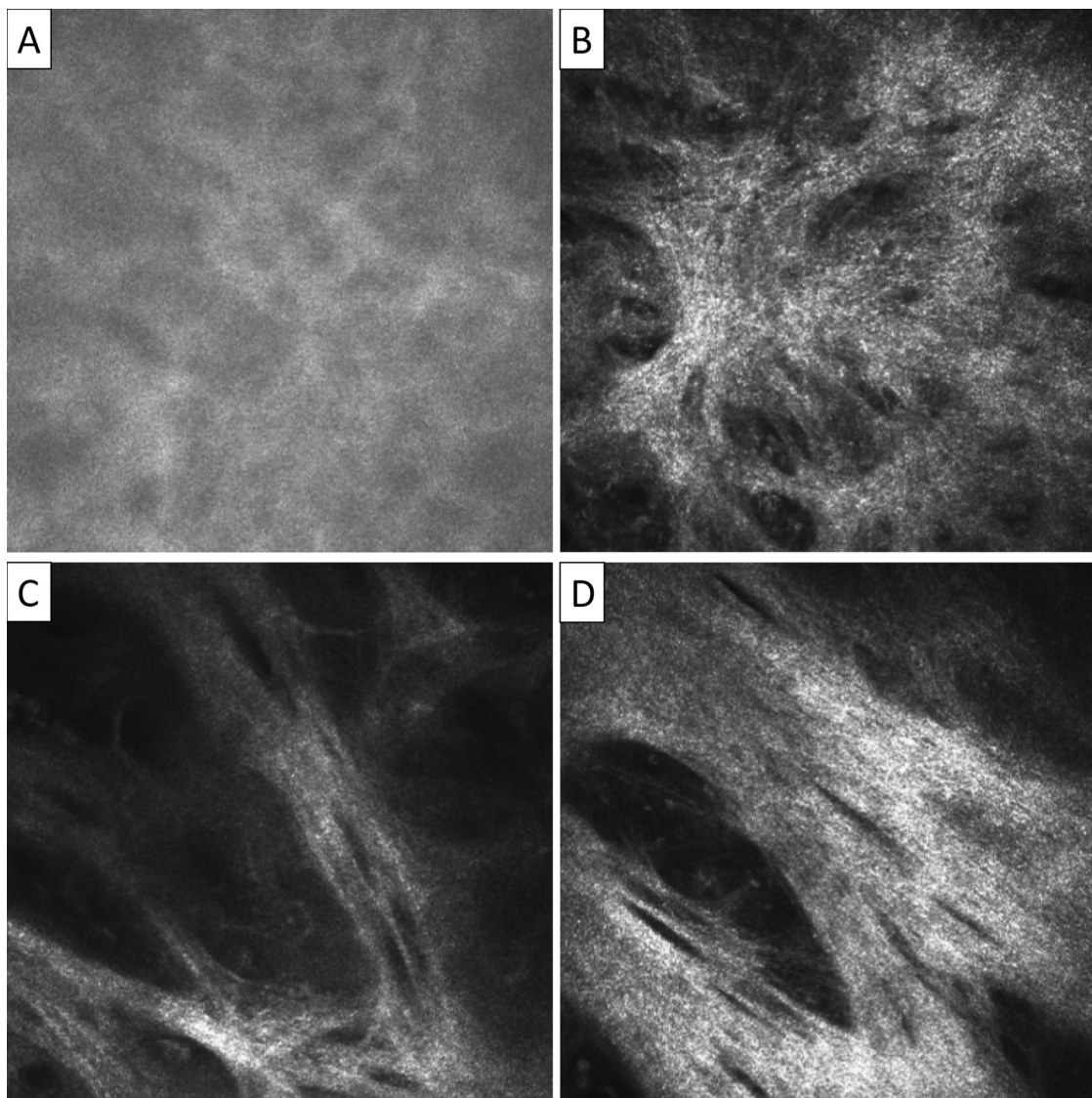
FIG 2 *In vivo* confocal microscopy grading system for conjunctival connective tissue organization. Images are 400 by 400 μm. (A) Normal: homogenous, amorphous appearance, with occasional, fine, wispy strands. (B) Grade 1: heterogenous appearance with poorly defined clumps or bands present. (C) Grade 2: clearly defined bands of tissue which constitute less than 50% of the area of the scan. (D) Grade 3: clearly defined bands or sheets of tissue which constitute 50% or more of the area of the scan and in which striations are present. If different grades of scarring are seen within a particular volume scan then the highest grade is recorded. The connective tissue which is graded needs to be separate from that associated with the vascular tissue; if this is not possible then the scan is considered ungradable.

cessfully detected for each target ranged from 313 to 359 for cases and 316 to 362 for controls.

Gene expression levels in relation to clinical findings. Cases with scarring had enriched gene expression compared to controls for the majority of transcripts measured, even after adjusting for age and sex (Table 1). This tended to be more marked in those cases with inflammation (increased: *INDO, TNFA, IL1B, S100A7, DEFB4A, CXCL5, SAA1, ARG, NOS2, MMP7, MMP9, MMP12, TIMP1,* and *CD83*; decreased: *MMP10, CFH,* and *SPARCL1*). There was also some evidence, but with less robust *P* values, that the expression of *IFNG* and *IL13RA2* were increased in cases with scarring.

After adjusting for age, sex, and the level of clinical scarring, a number of genes were also differentially expressed in those with clinical inflammation compared to those without inflammation (increased: *INDO, IL1B, DEFB4A, CXCL5, MMP7, MMP9, MMP12,* and *CD83*; decreased: *MMP10, SPARCL1,* and *CFH*).

Gene expression in relation to IVCM findings. The relationships between the gene expression results and the IVCM findings are presented in Table 2. Increasing IVCM connective tissue grade was associated with increased expression of *S100A7* (which also had the largest fold changes), *TIMP1,* and *CFH* and with decreased expression of *SPARCL1*. There was also some evidence, but with less-robust *P* values, that the connective tissue grade was associated with increased levels of *INDO, IL1B, SAA1, ARG1, MMP12,* and *CD83*.

The expression of a number of genes was correlated with the IVCM inflammatory infiltrate density. The correlation coefficient for *IL1B, S100A7,* and *DEFB4* was greater than $+0.40$

TABLE 1 Conjunctival gene expression in relation to clinical phenotype[a]

Gene[b]	TSI vs C		TS vs C		Inflamed vs noninflamed	
	FC	P	FC	P	FC	P
S100A7	5.54	<0.0001	3.2	<0.0001	1.3	0.11
SAA1	4.99	<0.0001	2.11	<0.0001	1.6	0.04
DEFB4A	4.72	<0.0001	1.42	0.01	2.46	<0.0001
CXCL5	3.89	<0.0001	1.18	0.43	3.4	<0.0001
MMP12	3.55	<0.0001	1.74	<0.0001	1.62	0.0002
INDO	3.29	<0.0001	1.5	<0.0001	1.96	<0.0001
MMP9	3.06	<0.0001	1.76	<0.0001	1.67	<0.0001
IL1B	2.57	<0.0001	1.59	<0.0001	1.48	0.0003
NOS2	2.16	<0.0001	1.27	0.005	1.38	0.007
MMP7	2.14	<0.0001	1.23	0.003	1.51	<0.0001
CD83	1.9	<0.0001	1.4	<0.0001	1.31	0.0004
IL13RA2	1.62	0.006	1.38	0.008	1.08	0.65
IL12B	1.52	0.02	1.29	0.04	1.22	0.23
TNFA	1.47	<0.0001	1.14	0.008	1.23	0.003
IL-10	1.46	0.2	0.64	0.03	1.27	0.42
TIMP1	1.46	<0.0001	1.4	<0.0001	0.93	0.3
IFNG	1.42	0.001	1.06	0.4	1.41	0.002
ARG1	1.3	0.01	1.25	0.0003	1.04	0.64
IL-13	1.22	0.3	1.17	0.19	1.09	0.64
CFH	0.75	<0.0001	1.13	0.005	0.68	<0.0001
MMP1	0.58	0.009	1.28	0.05	0.63	0.02
MMP10	0.38	<0.0001	0.86	0.02	0.54	<0.0001
SPARCL1	0.06	<0.0001	0.51	<0.0001	0.19	<0.0001

[a] TSI versus C and TS versus C values were adjusted for age and sex. Inflamed versus noninflamed values were adjusted for age, sex, and level of clinical scarring. Using a Bonferroni correction for 69 comparisons, a P value of <0.0007 would be considered significant. See the text for discussion. TSI, trachomatous scarring with inflammation; TS, trachomatous scarring without inflammation; FC, fold change.

[b] S100A7 = S100 calcium binding protein A7; SAA1 = serum amyloid A1; DEFB4A = defensin, beta 4A; CXCL5 = chemokine (C-X-C motif) ligand 5; MMP12 = matrix metalloproteinase 12; INDO = indoleamine-2,3-dioxygenase; MMP9 = matrix metalloproteinase 9; IL1B = interleukin-1; NOS2 = nitric oxide synthase 2, inducible; MMP7 = matrix metalloproteinase 7; CD83 = CD83 molecule; IL13RA2 = interleukin 13 receptor, alpha 2; IL12B = interleukin-12β; TNFA = tumor necrosis factor, alpha/beta; IL-10 = interleukin 10; TIMP1 = TIMP (tissue inhibitor of matrix metalloproteinase) matrix metalloproteinase inhibitor 1; IFNG = interferon gamma; ARG1 = arginase, liver; IL-13 = interleukin-13; CFH = complement factor H; MMP1 = matrix metalloproteinase 1; MMP10 = matrix metalloproteinase 10; SPARCL1 = SPARC (secreted protein, acidic, cysteine-rich)-like 1 (hevin).

(Fig. 3). For *SAA1*, *MMP9*, and *MMP12* it was between +0.25 and +0.35, and for *MMP10* and *SPARCL1* it was between −0.20 and −0.30. The presence of DFCs was associated with increased expression of *INDO* and *S100A7* and with decreased expression of *SPARCL1*.

Gene expression levels in relation to microbiology findings. Infection with *C. trachomatis* was associated with upregulated *IFNG*, *INDO*, and *NOS2* and with downregulated *MMP10* and *SPARCL1* (Table 3; note that only five participants had detectable *C. trachomatis* DNA). There was also some evidence, but with less-robust *P* values, that *C. trachomatis* infection was associated with increased *IL12B*, *MMP1*, *MMP7*, and *MMP10*. Nonchlamydial bacterial infection was associated with increased expression of *INDO*, *S100A7*, *DEFB4A*, and *MMP12* and with decreased expression of *MMP10*, *SPARCL1*, and *CFH* (Table 3). There was also some evidence that nonchlamydial bacterial infection was associated with increased *IL1B*, *IL-10*, *CXCL5*, *SAA1*, *MMP7*, and *MMP9*.

IVCM findings in relation to nonchlamydial bacterial infection status. A new combined analysis of previously published IVCM and microbiology data found that pathogenic organisms were associated with significantly more inflammatory cells (*P* < 0.0006) and with the presence of tissue edema (*P* = 0.02) (Table 4) (30, 32).

DISCUSSION

Previous studies have used gene expression measurement of conjunctival swab samples to study the pathogenesis of trachoma (6, 9, 14, 21, 22, 29, 46). However, this is the first study to systematically compare a large number of cases with trachomatous scarring and control subjects in which the scarring had not yet progressed to trichiasis. Cases with trichiasis are quite distinct from cases with scarring alone since they have a persistent foreign body abrading the globe stimulating inflammation and facilitating secondary infection (13). In the present study we have shown that scarring without trichiasis is associated with large fold changes in gene expression of various cytokines, chemokines, and other immunofibrogenic mediators, even though the scarring was generally mild to moderate.

We found that mild to moderate trachomatous scarring is strongly associated with a proinflammatory, innate immune response, which has marked antimicrobial properties, with increased expression of *S100A7*, *DEFB4A*, *CXCL5*, *IL1B*, *TNFA*, and *SAA1*. This is consistent with a transcriptome microarray study of end-stage scarring trachoma with trichiasis from Ethiopia (14). In both studies the expression of *S100A7* (psoriasin) showed the greatest fold increase between cases and controls. Psoriasin was initially identified as being upregulated in epithelial cells from psoriatic lesions (38). It is an antimicrobial peptide (AMP) and

TABLE 2 Gene expression levels in relation to *in vivo* confocal microscopy findings[a]

Gene	FC in gene expression with increasing connective tissue grade			IVCM infiltrate		FC in gene expression if dendritiform cells are present	
	Grade	FC	*P*	CC	*P*	FC	*P*
S100A7	1	1.00	<0.0001	0.43	<0.0001	2.03	0.0004
	2	1.64					
	3	3.76					
SAA1	1	1.00	0.002	0.28	<0.0001	1.80	0.03
	2	1.18					
	3	2.74					
DEFB4A	1	1.00	0.01	0.42	<0.0001	1.74	0.01
	2	0.84					
	3	1.60					
CXCL5	1	1.00	0.50	0.08	0.01	1.51	0.22
	2	1.25					
	3	1.40					
MMP12	1	1.00	0.004	0.31	<0.0001	1.36	0.05
	2	1.23					
	3	1.69					
INDO	1	1.00	0.004	0.16	<0.0001	1.71	0.0001
	2	1.17					
	3	1.60					
MMP9	1	1.00	0.01	0.28	<0.0001	1.19	0.19
	2	1.26					
	3	1.33					
IL1B	1	1.00	0.008	0.49	<0.0001	1.15	0.27
	2	1.18					
	3	1.45					
NOS2	1	1.00	0.14	0.15	0.02	1.28	0.07
	2	1.01					
	3	1.33					
MMP7	1	1.00	0.65	0.08	0.14	1.06	0.62
	2	1.02					
	3	1.12					
CD83	1	1.00	0.003	0.10	0.03	1.11	0.25
	2	1.21					
	3	1.13					
IL13RA2	1	1.00	0.79	0.14	0.005	0.92	0.65
	2	0.98					
	3	1.11					
IL12B	1	1.00	0.87	−0.09	0.01	0.90	0.59
	2	1.06					
	3	1.07					
TNFA	1	1.00	0.39	0.12	0.009	1.05	0.54
	2	1.07					
	3	1.04					

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TABLE 2 (Continued)

Gene	FC in gene expression with increasing connective tissue grade			IVCM infiltrate		FC in gene expression if dendritiform cells are present	
	Grade	FC	P	CC	P	FC	P
IL-10	1	1.00	0.15	0.13	0.0003	1.11	0.77
	2	0.86					
	3	0.49					
TIMP1	1	1.00	<0.0001	0.19	0.0005	1.09	0.27
	2	1.18					
	3	1.46					
IFNG	1	1.00	0.43	−0.12	0.0003	1.05	0.71
	2	0.91					
	3	0.89					
ARG	1	1.00	0.003	0.14	0.002	1.09	0.42
	2	1.24					
	3	1.17					
IL-13	1	1.00	0.13	0.001	0.98	0.92	0.27
	2	1.24					
	3	0.94					
CFH	1	1.00	0.0004	−0.09	0.004	0.93	0.30
	2	1.15					
	3	1.24					
MMP1	1	1.00	0.32	0.02	0.88	0.97	0.87
	2	1.06					
	3	1.40					
MMP10	1	1.00	0.02	−0.22	<0.0001	0.76	0.01
	2	1.14					
	3	0.85					
SPARCL1	1	1.00	<0.0001	−0.27	<0.0001	0.30	<0.0001
	2	0.87					
	3	0.67					

[a] Fold change (FC) values were adjusted for age and sex. Using a Bonferroni correction for 69 comparisons, a P value of <0.0007 would be considered significant. See the text for further discussion. IVCM, *in vivo* confocal microscopy; CC, correlation coefficient. See Table 1 for gene abbreviations.

therefore an important component of innate immune defense (56). Psoriasin is chemotactic for neutrophils and regulates neutrophil function by inducing the production of several cytokines, chemokines, and reactive oxygen species (34, 61). It has been shown to reduce *Escherichia coli* survival, the mechanism of which may be either through zinc sequestration or direct adherence with the bacteria (24, 36). It has more recently been shown to be upregulated at the ocular surface in response to *Staphylococcus aureus* and *Haemophilus influenzae* (23). Binding of *E. coli* flagellin by the Toll-like receptor 5 on keratinocytes is important in psoriasin induction, supporting its role as an innate immune molecule (2). The defensins are another group of AMPs found at the ocular surface, with β-defensins released by leukocytes and epithelial cells (26, 41). CXCL5 is a potent chemotaxin involved in neutrophil activation which is produced by epithelial cells (33, 56). Tumor necrosis factor alpha (TNF-α) and IL-1β are proinflammatory mediators which play a central role in mediating an innate immune response and have previously been found to be associated with active and scarring trachoma (3, 6, 9, 15, 22, 47).

Serum amyloid A, of which SAA1 is a main isoform, is an acute-phase protein which is raised in inflammatory diseases and has been shown to have antibacterial effects (19, 39).

The upregulation of these various proinflammatory, chemotactic, and antimicrobial mediators suggests an important role for the innate immune response in trachomatous scarring. This is consistent with the cellular paradigm of *Chlamydia trachomatis* pathogenesis. While there was some evidence of Th1 response in scarring (increased *IFNG* and *INDO* expression), there was no significant difference in *IL-13* expression, which might have suggested a Th2 response. There was some evidence that the expression of *IL13RA2*, which appears to act as a decoy receptor for IL-13 and opposes its function, was increased in cases compared to controls (60).

Many of the measured transcripts were upregulated in the presence of clinical inflammation. When scarred cases were compared to controls without scarring, the additional presence of inflammation in the cases tended to lead to more marked increases in gene expression. These observations are consistent with the hypothesis that episodes of inflammation are important in the
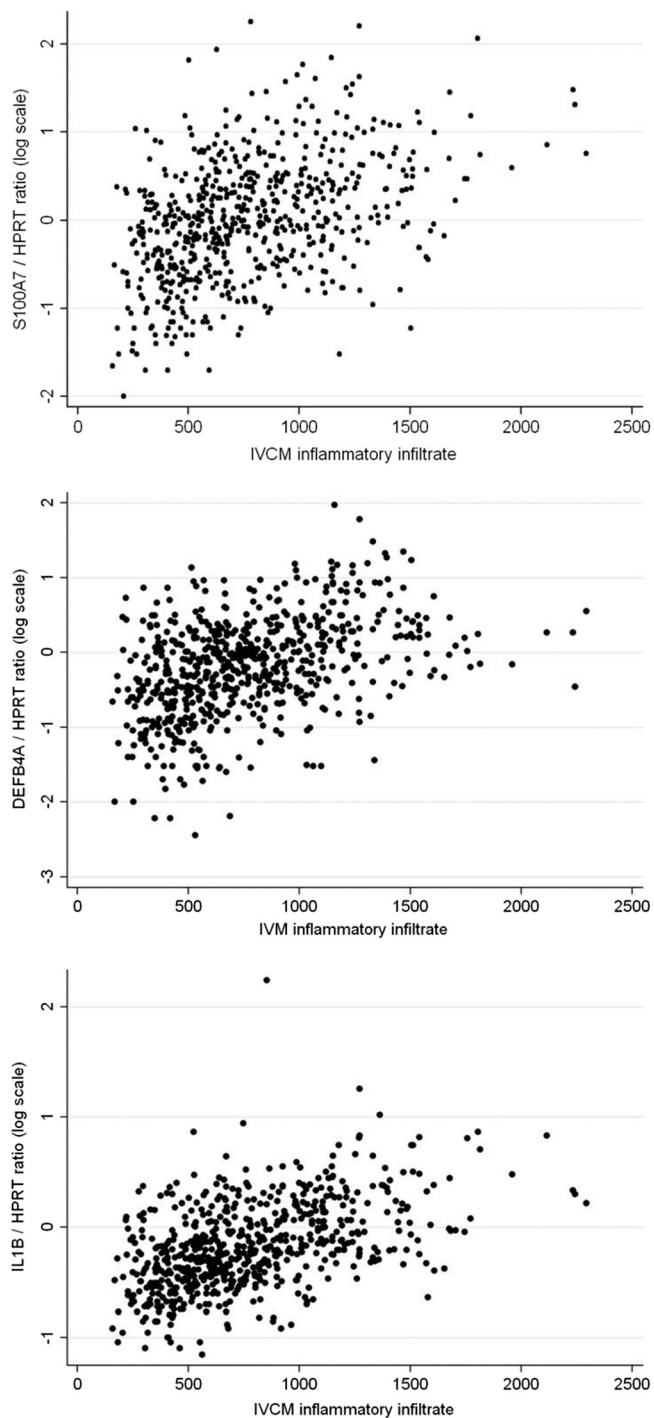
FIG 3 Gene expression levels relative to *HPRT1* of *S100A7*, *DEFB4A*, and *IL1B* by *in vivo* confocal microscopy inflammatory cell infiltrate.

pathogenesis of scarring and blindness. It is also interesting that a number of genes, for example, *S100A7*, *IL1B*, *SAA1*, and *MMP12*, showed large fold changes in gene expression even in relatively noninflamed cases with scarring compared to controls. These genes also showed a clearly progressive increase in expression with increasing IVCM connective tissue organization grade.

We found infection with *C. trachomatis* to be infrequent in

these adults with conjunctival scarring, with a similar detection rate to previous studies of trachomatous trichiasis (8, 11, 13). While caution is needed in interpreting these data because of the small numbers, individuals infected with *C. trachomatis* had gene expression profiles characteristic of *C. trachomatis* infection in children (9, 46). There was a typical Th1-dominated response, with upregulated *IFNG* and *IL12B* ($P = 0.05$) and also *INDO* and *NOS2* (both IFN-γ regulated) and some acute-phase reactants. Infection with other bacteria was more frequent, and many of the genes described above involved in innate immunity showed moderate to large increases in expression if a pathogenic organism was present. A previous study found that the presence of bacterial infection was associated with increased expression of *IL1B*, *TNF-α*, *MMP1*, *MMP9*, and *TIMP2* after trichiasis surgery (10). In the present study, bacterial infection was also found to be associated with an increased inflammatory infiltrate and tissue edema with IVCM. Overall, these studies support the hypothesis that nonchlamydial bacterial infection is important in driving the scarring process.

Matrix metalloproteinases are capable of degrading the protein components of the ECM and also have wide-ranging effects on inflammatory and immune responses (50, 58). Previous studies have implicated MMPs in the pathogenesis of trachomatous scarring, and this was further confirmed in the present study in a separate population (9, 10, 14, 20, 29, 44). *MMP7*, *MMP9*, *MMP12*, and *TIMP1* were upregulated in scarring and inflammation, whereas *MMP10* and *SPARCL1* were downregulated. SPARCL1 is a matricellular protein, which regulates the synthesis

TABLE 3 Gene expression in relation to microbiological infection status[a]

Gene	Chlamydial infection		Nonchlamydial bacterial infection	
	FC	P	FC[b]	P
S100A7	4.21	0.04	2.59	0.0006
SAA1	8.06	0.01	2.47	0.008
DEFB4A	4.82	0.02	3.56	<0.0001
CXCL5	8.2	0.07	4.15	0.002
MMP12	1.84	0.21	2.21	<0.0001
INDO	6.78	<0.0001	2.74	<0.0001
MMP9	1.57	0.28	1.73	0.002
IL1B	1.49	0.32	1.72	0.001
NOS2	4.5	0.0003	1.40	0.06
MMP7	3.01	0.002	1.52	0.003
CD83	1.30	0.34	1.27	0.05
IL13RA2	0.81	0.72	0.98	0.94
IL12B	3.10	0.05	1.16	0.56
TNFA	1.60	0.06	1.14	0.20
IL-10	1.29	0.81	3.30	0.005
TIMP1	0.98	0.94	1.09	0.36
IFNG	4.2	<0.0001	1.12	0.46
ARG	0.71	0.27	0.92	0.56
IL-13	0.26	0.02	0.66	0.13
CFH	0.64	0.03	0.67	<0.0001
MMP1	0.14	0.004	0.59	0.06
MMP10	0.34	0.002	0.46	<0.0001
SPARCL1	0.02	<0.0001	0.09	<0.0001

[a] Fold change (FC) values were adjusted for age and sex. Using a Bonferroni correction for 46 comparisons, a *P* value of <0.001 would be considered significant. See the text for further discussion. See Table 1 for gene abbreviations.
[b] Compared to no organism cultured.

TABLE 4 *In vivo* confocal microscopy parameters by nonchlamydial bacterial infection status[a]

IVCM parameter	No organism		Pathogenic organism		Adjusted increase[b]	P
	Mean	95% CI	Mean	95% CI		
Inflammatory infiltrate (no. of cells/mm^2)	731	694–769	972	(841–1102)	230	0.0006
Connective tissue organization score	1.04	0.97–1.11	1.31	(1.06–1.56)	0.12	0.40
	No.	%	No.	%	Adjusted OR[b]	
Dendritiform cells present	22	(6.0)	4	(14.3)	1.89	0.33
Tissue edema present	13	(3.6)	3	(10.7)	5.4	0.02

[a] CI, confidence interval; OR, odds ratio.
[b] Compared to no growth and adjusted for age and sex.

and turnover of the ECM (35, 54). The gene expression levels by clinical scarring grade were broadly consistent with the levels by IVCM connective tissue grade, and the expression levels by clinical inflammation were consistent with the IVCM inflammatory infiltrate correlation. The differential gene expression of these ECM modifiers was closely related to bacterial infection status as discussed above.

Strengths of this population-based study were that we studied a large number of cases with early scarring trachoma to analyze gene expression changes in relation to the clinical, IVCM, and microbiological status. There are, however, a number of limitations. Gene expression levels do not necessarily reflect the level of functional protein, exemplified by transforming growth factor β, which has significant posttranscriptional regulation. Conjunctival swabs obtain material from the tissue surface and so have a tendency to limit the observations to events in or near the epithelium. To address these two issues, we are currently comparing gene expression with immunohistochemical analysis of conjunctival biopsy tissue.

This study showed that mild to moderate trachomatous scarring is strongly associated with a proinflammatory, innate immune response and with differential expression of various modifiers of the ECM. We found no evidence for an active role for IL-13/Th2 responses at this stage of the disease. This is consistent with the findings from a microarray transcriptome study from Ethiopia on advanced trachomatous scarring (14). We were also able to use IVCM to show differential transcript levels according to connective tissue morphology and inflammatory cell infiltrate. A key determinant in the expression of many of these genes appears to be the presence of nonchlamydial bacterial infection which, as well as causing inflammation, may contribute to the scarring process.

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