Active Trachoma Is Associated with Increased Conjunctival Expression of *IL17A* and Profibrotic Cytokines[▽]

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The immunological basis of scarring trachoma is not well understood. It is unclear whether it is driven primarily through cell-mediated adaptive or epithelial-cell-derived innate responses. The purpose of this study was to investigate the expression of the inflammatory and fibrogenic mediators which may be involved. We conducted a cross-sectional survey of children living in an untreated trachoma-endemic community in Tanzania. The children were examined for signs of trachoma, and swabs were collected for bacteriological culture and RNA and DNA isolation. *Chlamydia trachomatis* was detected by the Amplicor PCR test. The expression of the following genes was measured by quantitative reverse transcription-PCR (RT-PCR): *S100A7*, *IL1B*, *IL17A*, *IL23A*, *CXCL5*, *CCL18*, *TLR2*, *NLRP3*, *KLRD1*, *CTGF*, and *MMP9*. Four hundred seventy children under the age of 10 years were included. Follicular trachoma (TF) was detected in 65 children (14%), *C. trachomatis* was detected in 25 (5%), and bacterial pathogens were cultured in 161 (34%). TF was associated with significantly increased expression of *S100A7*, *IL17A*, *CCL18*, *CXCL5*, and *CTGF*. Expression was increased further in the presence of papillary inflammation. Nonchlamydial bacterial infection was associated with increased expression of *IL17A*, *CXCL5*, *CCL18*, and *KLRD1*. *IL17A* expression was associated with increased expression of *S100A7*, *CXCL5*, *CCL18*, *KLRD1*, and *CTGF*. These data are consistent with a role for IL-17A in orchestrating the proinflammatory response in trachoma. Its activity may be promoted either as part of the cell-mediated response or through innate pathways. It may drive a range of proinflammatory factors leading to excessive tissue damage and repair involving fibrosis.

The basis of the immunopathology that characterizes clinically active trachoma and its link to the development of scarring disease of the conjunctiva remains to be elucidated. Recurrent chronic episodes of inflammation have been linked in long-term epidemiological studies to the subsequent development of scarring complications (16, 57, 59). The principal driver for this inflammatory scarring process in communities where trachoma is endemic is thought to be infection with *Chlamydia trachomatis*, although direct evidence is limited (59). The signs of inflammation and the detection of chlamydial infection may show only a relatively weak correlation, particularly in low-prevalence settings (8, 9, 60). This relationship weakens after the introduction of antibiotic treatment (28). Nonchlamydial bacterial pathogens have been associated with signs of active trachoma in a low-prevalence setting and in adults with established scarring (9, 10, 25). These observations raise the possibility that the chronic inflammatory reaction characterizing trachoma, which is the hypothesized basis for the development of scarring, may not always require an ongoing replicating chlamydial infection. It is possible that transient exposure to chlamydial antigens in an environment where the disease is endemic or to other bacteria may be sufficient stimuli to provoke ongoing active trachoma in individuals previously infected by *C. trachomatis*. (50).

Immunopathological correlates of active trachoma have been investigated using a number of different approaches, including peripheral blood mononuclear cell (PBMC) responses to chlamydial antigens (4), tear immunoglobulin and cytokine detection (5, 19, 48), conjunctival gene expression analysis by reverse transcription-PCR (RT-PCR) (6, 7), and, more recently, whole-conjunctival-transcriptome analysis (35). From this body of work, some broad conclusions emerge. First, the presence of detectable ocular *C. trachomatis* infection is associated with an activated Th1 response, characterized by increased expression of gamma interferon (IFN-γ) (7, 35). Animal models of genital chlamydial infection indicate that a Th1 response is probably necessary for the successful resolution of chlamydial infection, through IFN-γ-mediated mechanisms (43). Second, clinically active trachoma in children (irrespective of whether *C. trachomatis* is detected) and inflammation in scarred conjunctiva in adults are associated with a proinflammatory cytokine response (7, 11, 35). Third, recent information from whole-conjunctival-transcriptome analysis in children with active disease and adults with scarring trachoma found prominent innate immune responses (11, 35). Finally, there are some early pointers to the pathways involved in the development of cicatricial trachoma, with evidence of increased activity of

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various matrix metalloproteinases (MMP), which are involved in the regulation of the extracellular matrix (11). However, the field is far from clear; in particular, the processes involved in persistent active disease in childhood (even in the absence of infection) and how they link to the development of scarring are not understood. These outstanding questions are central to the pathophysiology of this disease, and without an adequate understanding of the processes involved, efforts to control the disease, particularly by vaccination, are likely to be held back.

We have conducted a cross-sectional population-based study of Tanzanian children living in an untreated trachoma-endemic community. We have previously reported the clinical and microbiological characteristics of this group, which had a community prevalence of follicular trachoma (TF) of 14% (9). Although *C. trachomatis* was detected in 5% of individuals, this was not associated with the presence of TF. In contrast, nonchlamydial bacterial pathogens were frequently associated with TF (9). This raises the possibility that in low-prevalence areas, at least, nonchlamydial bacterial pathogens may contribute to the inflammatory process.

Here we report the findings of a conjunctival gene expression study in the same children from this community, in whom we measured several factors that might be involved in the inflammatory response in active trachoma and its link to the development of scarring. The choice of targets focused on inflammatory-response mediators, pathogen pattern recognition receptors, and extracellular-matrix regulators, guided by results of previous whole-transcriptome studies (11, 35). The expression of interleukin-17A (*IL17A*) was elevated in the presence of follicular and papillary inflammation and was associated with coexpression of proinflammatory and profibrotic factors. It is possible that IL-17A and its related effects lie at the center of the pathogenesis of trachoma.

MATERIALS AND METHODS

Ethical approval. This study adhered to the tenets of the Declaration of Helsinki. The Tanzanian National Institute of Medical Research Ethics Committee, the Kilimanjaro Christian Medical Centre Ethics Committee, and the London School of Hygiene and Tropical Medicine Ethics Committee approved the study. Informed consent was documented in writing by the parent/guardian on behalf of each child before recruitment after the consent form was explained to the parent/guardian.

Study participants. The study was conducted in a single village in Siha District, Kilimanjaro region, northern Tanzania. After a census, all children under the age of 10 years who were present and for whom consent was given were enrolled into the study. This district had never received mass drug administration for trachoma control.

Clinical assessment and sample collection. The study procedures have been previously described (9). Briefly, participants were examined by an ophthalmologist and clinical signs graded using the 1981 WHO Trachoma Grading System (follicles, papillae, and conjunctival scarring [FPC]) (15). Follicular trachoma (TF) is equivalent to an FPC follicular score of 2 or 3 (F2/F3) (51). Three swab samples were collected from the anesthetized conjunctiva. A rayon-tipped swab sample was collected for microbiological culture from the inferior fornix, placed immediately into Amies-charcoal transport media (Sterilin, Caerphilly, United Kingdom), and kept at ambient temperature until processed in the laboratory later the same day. A second, Dacron polyester-tipped swab (Hardwood Products Company) was collected from the left upper tarsal conjunctiva for RNA extraction and placed directly into a tube containing 0.3 ml of RNA*later* (Life Technologies, Paisley, United Kingdom). A third swab was collected for detection of *C. trachomatis*. These two samples were collected in a standardized manner from parallel strips of tarsal conjunctiva and passed firmly four times

across the conjunctiva with a quarter turn between each pass. They were kept on ice packs until frozen later the same day at −80°C. The examiner changed gloves before examination and collection of swabs from each child. "Negative field controls" were collected after every 50 subjects by passing the swab through the air a few centimeters in front of a seated study participant. The population of the village was mass treated with single-dose oral azithromycin immediately after the survey, as per WHO guidelines.

Tests for conjunctival infection. Microbiology samples were inoculated onto blood and chocolate agar plates (usually less than 6 h from collection time) and incubated at 37°C for 48 h. Culture isolates were identified by standard microbiological techniques. *Staphylococcus epidermidis*, *Corynebacterium* spp., *Viridans* group *Streptococcus*, and *Bacillus* spp. were designated commensal organisms and excluded from the analysis of bacterial pathogens. To detect *C. trachomatis*, DNA was extracted from the dry swab using the QIAamp DNA minikit (Qiagen, Crawley, United Kingdom) and tested using the Amplicor CT/NG kit (Roche Molecular Systems, Branchburg, NJ), with previously described modifications (8, 24).

Quantitative RT-PCR for conjunctival gene expression. The abundance of 11 selected transcripts was estimated by quantitative RT-PCR. We selected a range of factors that are possibly involved in the inflammatory and fibrogenic processes that lead to scarring trachoma. The choice of targets was informed by two previously published conjunctival-transcriptome analyses conducted on samples from children with active trachoma in The Gambia, from matched healthy controls, and from Ethiopian adults with trachomatous trichiasis (11, 35). These targets fall broadly into several groups: proinflammatory cytokines, chemokines, or effector molecules (interleukin-1β [*IL1B*], interleukin-17A [*IL17A*], interleukin-23A [*IL23A*], psoriasin-1 [*S100A7*], chemokine [CXC] ligand-5 [*CXCL5*], and chemokine [C-C motif] ligand 18 [*CCL18*]), extracellular-matrix modifiers (matrix metalloproteinase 9 [*MMP9*] and connective tissue growth factor [*CTGF*]), pattern recognition receptors (NLR family pyrin domain-containing 3 [*NLRP3*] and Toll-like receptor 2 [*TLR2*]), and a natural killer (NK) cell marker (killer cell lectin-like receptor subfamily D, member 1 [*KLRD1*]).

Total RNA was extracted from the swab samples using the RNeasy microkit (Qiagen). Reverse transcription was performed using the 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, Crawley, United Kingdom), 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 using Beacon Designer 7.60 (Premier Biosoft International, Palo Alto, CA). The primer and probe concentration in each reaction mixture was 0.2 μM. The thermal cycle protocol used the following conditions: 95°C for 15 min, followed by 45 cycles of (i) denaturation at 94°C for 30 s and (ii) annealing and extension at 60°C for 30 s. Fluorescence data were acquired at the end of each cycle. The relative efficiencies of the component reactions were 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 upon request.

Analysis. Data were managed in Access (Microsoft) and analyzed in STATA 11 (StataCorp). The transcript abundances for genes of interest were standardized relative to that of *HPRT-1* in the same reaction using the $\Delta\Delta C_T$ method (where C_T is threshold cycle) and were normalized by $\log_{10}$ transformation (29). For several genes (*IL23A*, *CCL18*, *TLR2*, *NLRP3*, *KLRD1*), the target abundance was measured in singleplex assays, because it was not possible to multiplex with these other targets. Therefore, the quantitation for these targets relative to *HPRT* is based upon standard curve analysis. The relative levels of expression of genes of interest were compared using unpaired *t* tests for the presence of follicular inflammation (F2/F3), papillary inflammation (P2/P3), and bacterial pathogens on culture. Multivariable linear-regression models were fitted for the expression level of each target for the following potential explanatory variables: sex (female), age (in years), follicular inflammation (F2/F3), and the detection of a bacterial pathogen by culture. A stepwise selection process was performed to fit each model using likelihood ratio testing, with terms retained if the *P* value for omission was <0.2. Correlation coefficients and partial correlation coefficients (which take account of the lack of independence of gene expression) between *IL17A* and the other targets were calculated. To adjust for multiple comparisons, we calculated critical significance thresholds for each table using the conservative Bonferroni correction. Although we make several comparisons, these are unlikely to be truly independent of each other, as one would expect some of these genes to interact in biological networks.

TABLE 1. Clinical signs, *C. trachomatis* infection,
and bacterial infection[a]

Type of inflammation and clinical sign	Clinical grade		*C. trachomatis* infection		Bacterial infection	
	No. of children	%	No. of children	%[b]	No. of children	%[b]
Follicular						
Presence of follicular trachomatous inflammation						
No	405	86.2	21	5.2	121	29.9
Yes	65	13.8	4	6.2	40	61.5
FPC score of:						
F0	321	68.3	13	4.0	94	29.3
F1	84	17.9	8	9.5	27	32.1
F2	35	7.4	2	5.7	22	62.9
F3	30	6.4	2	6.7	18	60.0
Papillary						
Presence of intense trachomatous inflammation						
No	464	98.7	25	5.4	158	34.1
Yes	6	1.3	0	0.0	3	50.0
FPC score of:						
P0	369	78.5	16	4.3	112	30.4
P1	69	14.7	5	7.3	32	46.4
P2	26	5.5	4	15.4	14	53.9
P3	6	1.3	0	0.0	3	50.0

[a] Prevalences of clinical signs of active trachoma in 470 children in the study. The prevalences of *C. trachomatis* infection (as determined by Amplicor PCR) and other bacterial pathogens (as determined by culture, excluding commensal organisms) are given for TF and trachomatous inflammation scores of the simplified WHO system and the individual follicular (F) and papillary (P) inflammation grades of the FPC system.
[b] This percentage is for the number infected within each clinical disease grade.

RESULTS

Study participants and samples. The study participants have been described previously (9). Briefly, we examined 571/700 (81.5%) children <10 years of age living in the village. At recruitment, 75 (58%) were absent, 29 (23%) refused participation, and 25 (19%) were too young. Results of gene expression, bacteriology, and *C. trachomatis* PCR were available for a total of 470/571 (82.3%) children. When we compared the 470 children for whom results were obtained and all the other 230 children said to be living in the village, there was no difference in either the mean ages (4.85 versus 4.79 years; $P = 0.80$) or the proportions who were of male gender (49.2% versus 44.4%; $P = 0.23$). These 470 children were slightly younger than the 101 children who were examined but did not have all three sets of results (4.85 years versus 5.50; $P = 0.05$), but similar proportions were male (49.2% versus 41.6%; $P = 0.17$). All subsequent analyses were limited to the 470 children with all laboratory results available.

Clinical signs and infection. The relationship between the clinical signs and both *C. trachomatis* infections and other bacterial infections in this sample has been previously reported in detail (9). Briefly, TF was diagnosed in 65 (13.7%) children. The frequencies of the FPC grading system follicular (F) and papillary (P) inflammation scores are shown in Table 1. *C. trachomatis* DNA was detected by Amplicor CT/NG PCR in 25 (5.3%) samples, and bacterial pathogens were cultured from

TABLE 2. Gene expression level by follicular inflammation,
papillary inflammation, and bacterial infection[a]

Target	Follicular inflammation (F2/F3)		Papillary inflammation (P2/P3)		Bacterial infection	
	FC	*P* value	FC	*P* value	FC	*P* value
S100A7	2.49	<0.0001	2.61	0.0002	1.51	0.003
IL1B	1.20	0.33	1.24	0.39	1.28	0.07
IL17A	3.32	<0.0001	5.53	<0.0001	2.43	<0.0001
IL23A	1.23	0.06	1.17	0.30	1.25	0.005
CXCL5	4.16	<0.0001	9.52	<0.0001	2.19	<0.0001
CCL18	3.38	<0.0001	7.82	<0.0001	2.66	<0.0001
NLRP3	1.18	0.05	1.21	0.09	1.23	0.0005
KLRD1	1.29	0.01	1.11	0.45	1.61	<0.0001
TLR2	1.20	0.004	1.19	0.04	1.16	0.0012
CTGF	2.22	<0.0001	2.52	<0.0001	1.42	0.0001
MMP9	1.22	0.04	1.23	0.12	1.05	0.48

[a] Gene expression levels were determined for children with follicular inflammation, papillary inflammation, and bacterial infection (by culture, with commensal organisms excluded). The fold changes (FC) in gene expression are derived from comparisons of children with follicular inflammation (F2 or F3) versus no follicular trachoma (F0 or F1), papillary inflammation (P2 or P3) versus no papillary inflammation (P0 or P1), and bacterial infection versus no bacterial infection. *P* values are for the unpaired *t* test. Using the Bonferroni correction for multiple comparisons, the critical significance threshold level is a *P* value of <0.0015.

161 (34.3%) eyes. *Streptococcus pneumoniae* and *Haemophilus influenzae* accounted for 89.7% of the pathogenic bacterial isolates (9). All negative field controls tested negative by Amplicor *C. trachomatis* PCR. The frequencies of *C. trachomatis* PCR-positive and cultured bacterial pathogens by clinical grade are shown in Table 1. There was no association between the presence of TF and the detection of *C. trachomatis* (odds ratio [OR], 1.20; 95% confidence interval [95%CI], 0.40 to 3.61; $P = 0.75$). There was, however, a significant association between the presence of TF and the culture of a bacterial pathogen (OR, 4.69; 95%CI, 2.31 to 9.52; $P < 0.001$).

Gene expression in active trachoma and infection. The levels of expression of the genes relative to the presence of clinical features of trachomatous conjunctival inflammation are shown in Table 2. Significantly increased expression of *S100A7*, *IL17A*, *CXCL5*, *CCL18*, and *CTGF* was found in the presence of both follicular inflammation (F2/F3) and papillary inflammation (P2/P3); the critical significance threshold was a *P* of 0.0015 (Bonferroni correction for 33 comparisons). The fold changes were greater in the presence of papillary inflammation than in the presence of follicular inflammation for each of these five targets. There were no differences in gene expression in the presence of *C. trachomatis* detected by Amplicor CT/NG (data not shown), possibly due to the small numbers of infections. In contrast, the presence of a nonchlamydial bacterial pathogen was associated with significantly increased expression of *IL17A*, *CXCL5*, *CCL18*, *TLR2*, *NLRP3*, *KLRD1*, and *CTGF*; there were also borderline increases in the expression of *S100A7* and *IL23A*. Multivariable linear-regression models for the expression of each target indicated that, after adjustment for gender, age, and the presence of bacterial pathogens, follicular trachoma (F2/F3) was significantly associated with increased expression of *S100A7*, *IL17A*, *CXCL5*, *CCL18*, and *CTGF* (Table 3). The correlation between the expression levels of *IL17A* and the other targets is shown in Table 4.

TABLE 3. Multivariable linear-regression models for the expression of each target[a]

Target and parameter	FC	P value
S100A7		
Gender (female)	0.68	0.003
Age (yr)	1.04	0.11
Follicular trachoma (F2/F3)	2.39	<0.0001
Bacterial infection	1.37	0.03
IL1B		
Gender (female)		
Age (yr)	0.96	0.13
Follicular trachoma (F2/F3)		
Bacterial infection	1.22	0.16
IL17A		
Gender (female)	1.15	0.18
Age (yr)	0.92	<0.0001
Follicular Trachoma (F2/F3)	2.53	<0.0001
Bacterial infection	1.83	<0.0001
IL23A		
Gender (female)	0.86	0.04
Age (yr)		
Follicular trachoma (F2/F3)	1.17	0.17
Bacterial infection	1.22	0.02
CXCL5		
Gender (female)		
Age (yr)	0.94	0.06
Follicular trachoma (F2/F3)	3.40	<0.0001
Bacterial infection	1.64	0.008
CCL18		
Gender (female)		
Age (yr)	0.93	0.0004
Follicular trachoma (F2/F3)	2.55	<0.0001
Bacterial infection	2.05	<0.0001
NLRP3		
Gender (female)		
Age (yr)	0.98	0.02
Follicular trachoma (F2/F3)		
Bacterial infection	1.19	0.004
KLRD1		
Gender (female)		
Age (yr)	0.91	<0.0001
Follicular trachoma (F2/F3)		
Bacterial infection	1.42	<0.0001
TLR2		
Gender (female)	0.95	0.19
Age (yr)	0.99	0.15
Follicular trachoma (F2/F3)	1.16	0.02
Bacterial infection	1.11	0.02
CTGF		
Gender (female)		
Age (yr)	0.97	0.03
Follicular trachoma (F2/F3)	2.04	<0.0001
Bacterial infection	1.20	0.04
MMP9		
Gender (female)		
Age (yr)		
Follicular trachoma (F2/F3)	0.82	0.04
Bacterial infection		

[a] Terms were retained in the model if their P value was <0.2, by likelihood ratio testing. FC, fold changes, which were derived from the models. The specific comparisons within these terms of the model were female versus male, age as a continuous variable in years, follicular trachoma (F2 or F3) versus no follicular trachoma (F0 or F1), and bacterial infection versus no bacterial infection. Using the Bonferroni correction for multiple comparisons, the critical significance threshold level is a P value of <0.0016.

TABLE 4. Correlation and partial correlation coefficients for the expression of *IL17A* compared to that of the other measured targets[a]

Target	Correlation coefficient	Partial correlation coefficient	P value
S100A7	0.3392	0.1880	0.0001
IL1B	0.1921	−0.0637	0.18
IL23A	0.2095	−0.0043	0.93
CXCL5	0.5185	0.3191	<0.0001
CCL18	0.5201	0.2215	<0.0001
NLRP3	0.1925	0.0144	0.76
KLRD1	0.2454	0.1870	0.0001
TLR2	0.3233	0.0157	0.74
CTGF	0.4910	0.2933	<0.0001
MMP9	−0.1590	−0.1292	0.006

[a] The P value refers to the partial correlation coefficient. Using the Bonferroni correction for multiple comparisons, the critical significance threshold level is a P value of <0.005.

DISCUSSION

At the epidemiological level, trachomatous conjunctival inflammation in childhood is linked to subsequent scarring (16, 57, 59). The molecular basis for this process, which has received relatively little attention in trachoma research due to the difficulty of conducting such studies, is not understood. In this cross-sectional population-based study, we found that follicular and papillary inflammations were associated with increased expression of *IL17A*, *S100A7*, *CXCL5*, *CCL18*, and *CTGF* and that nonchlamydial bacterial infection was associated with increased expression of *IL17A*, *CXCL5*, *CCL18*, and *KLDR1*.

Animal and human studies suggest that successful resolution of *C. trachomatis* infection probably involves an IFN-γ-dependent Th1 cell-mediated immune (CMI) response (43, 44). However, the immunopathogenic basis of the scarring complications of ocular and genital tract infections is not well understood (14). Several theories for pathogenesis (none of which entirely fit the available data), which fall into two broad paradigms, immunological (cell mediated) and cellular (innate) (49), have been proposed.

The immunological paradigm suggests that immunopathology results from various antigen-specific CMI responses (14, 50). In a classical delayed-type hypersensitivity reaction, Th1 responses are enhanced on rechallenge. Models of repeated *C. trachomatis* infection that develop scarring are characterized by T lymphocytes and Th1 cytokine production, possibly linking these to tissue-damaging inflammation (53). However, people with established trachomatous scarring and current infection have weaker PBMC Th1 responses when stimulated with *C. trachomatis* antigens, leading some to reject this mechanism (23, 49). Th2-dominated responses, mediated through IL-13 and alternatively activated macrophages (AAMΦ), are linked to several scarring diseases, for example, ocular cicatricial pemphigoid and hepatic fibrosis in schistosomiasis (47, 61). However, there is currently little evidence for this mechanism in trachoma. PBMCs from cases with severe scarring more frequently produced IL-4 and less frequently produced IFN-γ than PBMCs from controls when they were stimulated with *C. trachomatis* antigens (23). However, conjunctival-transcriptome profiling of adults with established scarring found lower

IL13 expression, a marked increase in nitric oxide synthase 2A (*NOS2A*) expression, and only a slight increase in arginase-1 (*ARG1*) expression, consistent with predominantly classically activated macrophage activity (11, 24, 62). Similarly, IL-13 has been detected less frequently in tear fluid from individuals with trachomatous scarring than from controls (48). Previous studies of active trachoma in children have examined factors linked to a Th2 response, with inconclusive results. *IL4* expression has been detected more frequently in children with active trachoma and chlamydial infection than in control children, although a separate study found no difference in the tear IL-4 concentrations (7, 48). Conjunctival-transcriptome analysis of children with active chlamydial infection found widespread evidence of IFN-γ activity but relatively little to suggest Th2 activity (35).

In the cellular paradigm, inflammatory responses are initiated and sustained by the innate immune system through pattern recognition receptors (PRRs), particularly on epithelial cells (14, 49). *In vitro* infection of epithelial cell cultures with *C. trachomatis* results in the production of several proinflammatory cytokines (45). Microarray transcriptome data indicate prominent innate immune responses in trachoma, consistent with this cellular paradigm (11, 35). However, neither of these two paradigms provides a satisfactory explanation for the pathogenesis of trachoma on its own; they are not mutually exclusive and have common characteristics.

Expression of *IL17A* was enriched in the presence of signs of active trachoma and correlated with the expression of *CXCL5*, *S100A7*, *CCL18*, and *CTGF*. IL-17A, a member of the interleukin-17 superfamily, is a cytokine which contributes not only to defense, especially against extracellular bacteria, but also to immune-mediated pathology in various diseases (37). It was originally described to arise from a distinct class of T-helper cells (Th17), which develop under the combined stimulation of transforming growth factor β (TGF-β), IL-1β, IL-6, and IL-23 (37). Other cell types also produce IL-17A: γδ T cells, NK cells, macrophages, and possibly neutrophils and epithelial cells (46). IL-17A has profound proinflammatory effects that can lead to extensive tissue damage, particularly mediated through neutrophils (37). These include the induction of chemokines, S100 proteins, and matrix metalloproteinases (37). The stimulation of Th17 cells to produce IL-17A is antigen specific. However, IL-17A production may also occur through innate pathways, with signaling through receptors such as TLR2 leading to IL-17A production by Th17 cells, γδ T cells, and NK cells (46). The regulation of IL-17A and Th17 cells is complex; they appear negatively regulated by Th1 (IFN-γ), Th2 (IL-4), and Treg (IL-10, TGF-β) cells. The balance between Th1 and Th17 appears critical, with excessive Th17 activity leading to immunopathology. Recent observations about IL-17A and Th17 cells in another intracellular bacterial infection, *Mycobacterium tuberculosis*, may be instructive and may conceivably have parallels in chlamydial infection (52). In a murine model of *M. tuberculosis* lung infection, subsequent repeated vaccination with BCG led to extensive tissue damage by neutrophils, and this damage was IL-17 dependent and was without the loss of Th1-mediated immunity to *M. tuberculosis*. Repeated exposure to BCG antigen led to amplification in the Th17 response, which became refractory to regulation by IFN-γ/Th1. It is possible that in trachoma a similar imbalance

between Th1 and Th17 responses may arise from repeated *C. trachomatis* infection or exposure to chlamydial antigens. While the resolution of infection may be unaffected, the tissue may experience augmented inflammatory responses not only to chlamydia but also to nonspecific triggers, such as other bacteria, through innate response mechanisms. It is interesting to note that *IL17A* expression was increased in children with bacterial infection. These observations suggest a possible role for IL-17A in the development of the inflammatory pathology in trachoma.

Psoriasin (S100A7), a member of the S100 family, was initially identified in psoriatic skin lesions and is closely associated with inflammatory epithelial pathology (17, 30). S100A7 production is triggered by some bacterial components through PRRs and by proinflammatory cytokines (2, 22, 56). It is constitutively expressed in ocular surface epithelium and increases in corneal epithelial cell culture upon stimulation with bacterial antigens and proinflammatory cytokines (IL-1β, tumor necrosis factor alpha [TNF-α]) (18). Psoriasin is an important antimicrobial peptide protecting epithelial surfaces from bacterial infection (20, 32). The antimicrobial action is possibly mediated through pore formation or zinc sequestration (20, 33). S100A7 is chemotactic for both neutrophils and T lymphocytes and may be important in driving the local innate immune response (27, 58). It stimulates neutrophils to produce proinflammatory cytokines and reactive oxygen species and induces degranulation (64). Others have suggested that S100A7 stimulates keratinocytes to produce cytokines that promote a Th1/Th17 response (58). Stimulation of keratinocytes with a combination of IL-17, IL-22, and TNF-α (produced in a Th1/Th17 response) increased S100A7 production, which may form a feedback loop, amplifying inflammation (58). Previously, we found increased *S100A7* expression in adults with scarring trachoma, both with and without concurrent conjunctival inflammation (11) and by microarray analysis of active trachoma in children (35). Interestingly, *S100A7* is also expressed at increased levels in the conjunctivas of individuals with recurrent trichiasis following surgery, after controlling for preoperative disease severity (unpublished data). Increased *S100A7* expression in active trachoma, independently of bacterial infection, suggests a potential role for provoking and sustaining the inflammatory clinical phenotype, which warrants investigation.

Expression of *CXCL5* was significantly increased in active trachoma, independently of bacterial infection. Similar increased expression has previously been found in scarring trachoma (11) and by microarray analysis of active trachoma in children (35). CXCL5 induces neutrophil chemotaxis and activation; it also induces fibroblast precursor chemotaxis and differentiation (36). Therefore, it could conceivably contribute to inflammation and fibrosis. Epithelial cells are probably the main source of CXCL5, although macrophages and fibroblasts may also contribute. Epithelial cell lines have been shown to produce CXCL5 in response to chlamydial infection, which led to neutrophil chemotaxis (63). Animal models of pulmonary inflammation have demonstrated CXCL5 production in response to bacterial antigens through TLR4-dependent signaling (26). It is therefore possible that in the context of cicatricial trachoma, other bacterial pathogens stimulate CXCL5 production directly or indirectly through other cytokines (IL-1β,

TNF-α) or products of extracellular-matrix breakdown (e.g., actin) (54).

The Th2 chemokine CCL18 had the largest increase in expression of the CC chemokines detected by microarray analysis of active disease with concurrent chlamydial infection (35). We found that *CCL18* expression significantly increased in association with both follicular trachoma and bacterial infection. CCL18 is produced by AAMΦ in response to Th2 cytokines. It is closely associated with tissue fibrosis in sarcoidosis, systemic sclerosis, and idiopathic pulmonary fibrosis and is a reliable biomarker for progressive pulmonary fibrosis (31, 40–42). CCL18 promotes lymphocyte chemotaxis and the development of a T-regulatory phenotype, with production of TGF-β and IL-10, but not IFN-γ or IL-4 (12). Finally, CCL18 markedly increases collagen type 1 production by fibroblasts *in vitro* (39). The finding of a consistent association in two separate populations between active trachoma and increased *CCL18* expression suggests a potential role for this chemokine in linking inflammatory trachoma in childhood to the development of scarring. In view of the reported link between Th2 responses and CCL18, it is possible that this chemokine may reflect some subtle underlying Th2 activity in active trachoma that is obscured by the more florid IFN-γ response.

There are several well-characterized mediators and pathways in human scarring diseases (61). The archetypal profibrotic molecule is TGF-β. However, there are other potent profibrotic factors, such as CTGF, platelet-derived growth factor (PDGF), and IL-13. The observation that *CTGF* expression is significantly increased in active trachoma may indicate another mechanistic link between the childhood disease and the development of scarring. CTGF has previously been found in conjunctival stromal macrophages in tissue from children with active trachoma (3). CTGF is an important profibrotic mediator in several fibrotic disease processes, including renal and hepatic fibrosis and systemic sclerosis (1, 13, 21). Its expression is regulated by TGF-β (38). It has direct profibrotic effects and also mediates the effect of TGF-β; the two act synergistically (38, 55). There is a suggestion that for persistent tissue fibrosis to develop, CTGF is required in addition to TGF-β; in the absence of CTGF, TGF-β-induced fibrosis may have a reversible quality (34). CTGF stimulates fibroblasts to migrate, proliferate, and produce extracellular matrix. It also stimulates the transition from epithelial cells to mesenchymal cells, the importance of which is unknown in scarring trachoma. The study of TGF-β in trachoma has proved challenging because of its complex posttranscriptional regulation. A previous gene expression study found *TGFβ2* to be constitutively expressed in the conjunctiva across disease groups (7). *CTGF* expression may therefore offer an indirect way of investigating TGF-β activity.

The association between bacterial pathogens and the increased expression of several factors in this study is noteworthy and has previously received limited attention. The data in this study suggest that these other infections appear to have an effect on the expression of factors which may contribute to the scarring process. It is possible that repeated conjunctival exposure to *C. trachomatis* leads to altered, excessively inflammatory responses to other pathogens, resulting in a clinical phenotype that not only is indistinguishable from follicular conjunctivitis caused by *C. trachomatis* but also is associated

with a similar profibrotic inflammatory response. We think that this is an area that warrants further investigation.

Caution needs to be exercised in the interpretation of cross-sectional studies when results are extrapolated to longitudinal disease processes. Second, we can report only associations, but these are useful for the development of new hypotheses and further investigation. Longitudinal studies are needed to investigate potential causal links between specific immunological responses and progressive scarring disease. Gene expression analysis of conjunctival surface swab samples has the advantage that it is an acceptable, noninvasive investigation; however, it may yield a biased cell population, and the presence of a functional protein can only be inferred. In this study population, the burden of chlamydial infection was low. This limits what this data set can tell us about the responses to the infection itself; however, it is helpful in the exploration of the potential basis of the active trachoma phenotype after the infection has resolved.

In summary, we found evidence of enriched expression of *IL17A* in association with the signs of active trachoma. This was associated with increased expression of *S100A7*, *CXCL5*, *CCL18*, and *CTGF*. This raises the possibility of IL-17A and Th17 activity being important in the development of the inflammatory pathology that leads to scarring trachoma. The increased chemokine responses are likely to result in increased neutrophil chemotaxis and activation. This would lead to greater tissue damage, which, if prolonged, would result in fibrosis as part of the wound healing response. IL-17A and Th17 cells provide a link between the adaptive and innate responses in chlamydial infection, possibly providing a unifying explanation for the two disease paradigms and explaining how this organism causes disease.

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