

## Evidence for a Predominant Proinflammatory Conjunctival Cytokine Response in Individuals with Trachoma

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**Immune responses to *Chlamydia trachomatis* infection in trachoma do not protect against reinfection or the development of scarring and blindness. In addition, the immunoregulatory contribution of cytokines to the development of conjunctival histopathology or protection is undefined. In this study, conjunctival cytokine mRNA transcripts were compared among subgroups of chlamydia infection status and ocular disease presentations of 50 individuals from an area where trachoma is endemic. There was a significant association of elevated interleukin (IL)-1 $\beta$ , transforming growth factor  $\beta$ 1, and tumor necrosis factor alpha transcripts with infection, follicular inflammation, and scarring. Both gamma interferon (IFN- $\gamma$ ) and IL-2 transcripts were significantly associated with infection; slightly elevated IL-2 levels were found in inflammatory disease. High IFN- $\gamma$  transcript levels were present with follicles and inflammatory disease and to a lesser extent with inflammatory scarring. The role of IFN- $\gamma$  in protection from infection or disease was not apparent from this study, since transcripts were frequently present in both chlamydial infection and disease. IL-12 (p40) transcripts were elevated in adults and children in association with follicular inflammation but not with scarring. IL-4, IL-5, and IL-10 transcripts were not detected in any samples. In conclusion, *C. trachomatis* infection stimulates local cytokines which favor a strong cell-mediated and proinflammatory response in both the early and later manifestations of trachoma. In addition, cytokine transcript levels that were associated with disease but no infection were characteristically lower overall than when chlamydia was present.**

Trachoma, which is caused by *Chlamydia trachomatis*, is the leading infectious cause of blindness in the world, and cases of blinding trachoma are predicted to reach 12 million within the next 30 years (39). Although many individuals living in areas where the disease is hyperendemic experience chlamydial ocular infection, a substantial proportion escape the sequelae of reinfection, namely, trichiasis and blindness. Epidemiological studies have indicated that sociologic and environmental risk factors do not totally explain this finding and that there seems to be a differential response to reinfection in subsets of individuals which is either protective or pathologic (47). A delayed-type hypersensitivity response to reinfection is thought to participate in the development of histopathology (30). This differential response to reinfection suggests that an immunointervention strategy could modulate pathology or protect against chlamydia infection. However, vaccine efforts have not made significant strides, in large part because there is little information about local cell-mediated immune response during natural infection with *C. trachomatis*.

The importance of T cells and natural killer cells in recovery from chlamydia infection has been shown in vitro and in animal infection models. Recently, several studies have demonstrated cytotoxic-lymphocyte lysis of chlamydia-infected target cells and that adoptive transfer of cytotoxic lymphocytes reduced infection in mice (4, 43). Major histocompatibility complex class II-restricted T-cell responses were necessary for the development of protection in a mouse genital model (31). In the same animal model, adoptive transfer of splenic CD4<sup>+</sup> and

CD8<sup>+</sup> T cells indicated that CD4<sup>+</sup> rather than CD8<sup>+</sup> T cells were protective (45). Enzyme-linked immunosorbent assay measurements for interleukin-2 (IL-2), IL-6, and gamma interferon (IFN- $\gamma$ ) were elevated in primary and secondary infections, with IL-4 and IL-5 extremely low or not detectable, respectively. In other mouse studies, IFN was important in early chlamydia clearance and survival and was produced in part by natural killer cells (49). However, tissue culture studies have indicated that IFN- $\gamma$  might also play a role in the persistence of infection and continued disease (5, 36).

Studies of human cell-mediated immune response in chlamydia infection are few. In one report, individuals with trachomatous scarring were nonresponsive in terms of their mononuclear cell proliferative response to elementary bodies and IFN- $\gamma$  levels were low (23). In follicular inflammation, the proliferative response was greater when infection was resolved than when clinical signs persisted, but IFN- $\gamma$  levels were similar in both groups (2).

In other infectious diseases, such as leprosy, tuberculosis, human immunodeficiency virus infection, leishmaniasis, and schistosomiasis, certain cytokine patterns have been associated with disease severity or protection (3, 26, 51, 52). This information has led not only to a better understanding of the contribution of cytokines to immunity or pathogenesis but also to new strategies for therapeutic intervention (35). We chose as a study population people who live in a setting where trachoma is hyperendemic and *C. trachomatis* reinfection occurs and who are exposed to similar environmental and societal risk factors. To better understand the local cellular response, mRNA transcripts from selected cytokines with known effector functions characteristic of the Th1-Th2 paradigm and those which are inflammatory or involved with fibrogenesis in other diseases were measured and compared with the presence of *C. trach-*

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TABLE 1. Outer and nested primers used in the PCR-EIA

Target name (reference[s])	Outer primers <sup>a</sup>	Nested primers for probe <sup>a,b</sup>
β-Actin (32)	TGGATGATGATATCGCCGCG CATCTTCTCGCGGTTGGCCT	GCATGGGTGAGAAGGATTAATATG CACGCAGCTCATTGTAGAAAGGTG
IFN-γ (19)	GCTGTTACTGCCAGACCCAT CATGTCTTCTTGATGGTCTC	GCAGAAAACCTTAAGAAAT CTTTGGATGCTCTGGTC
IL-1β (12)	TGGCAATGAGGATGACTTGT CCATGTGTGCAAGAAGATAG	GCTCCTTCCAGGACCTGGAC GTGCTCAGGTCATTCTCCT
IL-2 (28)	GATGCAACTCCTGTCTTG TATACGTTGATATTGCT	CACAGCTACAACCTGGAG TTAGHACTTCCAGAG
IL-4 (1)	ACTTTGAACAGCCTCACAGAC GATCGTCTTTAGCCTTTCC	GAGTTGACCTAACAGAC CTGTGGAACCTGCTGTGCAGT
IL-5 (10)	ATCCCCACAGAAATTCCC TCCGTCTTTCTTCCACAC	GCACTGCTTTTCTACTC AATAGTCTTTCCACAGTA
IL-6 (22)	GTACATCCTCGACGGCAT GTATTGCATCTAGATTC	GAGACATGTAACAAGAGT CTCACTACTCTCCAAATCT
IL-10 (46)	CAGTCTGAGAACAGCTGC CATTCTTCACCTGCTCCA	CAGGCCTGCCTAACAT GATCATCTCAGACAAGGCT
IL-12 p40 (50)	CCAAGAACTTGCAGC TGGGTCTATTCCGTTGTGTC	GTCATCTTGGAGCGAAT TGCGGCAGATGACCGTGG
TGF-β1 (40)	GTCACCCGCGTGCTAATG CACGATCATGTTGACAC	ATATATGTTCTTCAAC GTCAATGTACAGATGCCGC
TNF-α (33, 34)	TTCTGCCTGCACCTTGGAG AAGAGGACCTGGGAGATGAGGTA	GAACCCCGAGTGACAAGCCT GTCAATGTACAGCTGCCGC
CD3-δ (52)	CTGGACCTGGGAAAACGCATC GTACTGAGCATCATCTCGATC	GCAAGTTCATTATCGAATG <sup>c</sup> CAACAGAGCTTGTGTGTTCCG

<sup>a</sup> All primers are 5' to 3', and the sense oligonucleotide is above the antisense oligonucleotide.

<sup>b</sup> The nested sense primer is preceded at the 5' end by the T7 RNA polymerase promoter sequence TTAATACGACTCACTATAGGGT.

<sup>c</sup> Nested primer sequences were obtained by sequencing a clone derived from the outer fragment generated by the target primers.

*matris* infection and with various clinical manifestations of trachoma.

#### MATERIALS AND METHODS

**Clinical samples.** As part of a village-wide survey to evaluate antibiotic efficacy, human tarsal conjunctival swab samples were obtained before treatment from 129 individuals living in two Tanzanian villages where trachoma is endemic. The conjunctivae were examined with a 2.5× loupe and graded for trachoma by using the World Health Organization grading scheme (15). The following categories were used: TI, intense follicular inflammation obscuring 50% of the deep tarsal vessels; TF, the presence of five or more follicles of >0.5 mm with no signs of TI; TC, the presence of scars alone; TIC, scarring and inflammation; NS, no signs of trachoma in either eye. A subgroup of 50 samples was chosen for further analysis by selecting at random 50% infected (positive for chlamydia by PCR) and 50% uninfected samples. We attempted to sample an equal number of persons in each disease subcategory; thus, these samples did not constitute a representative sample of disease and chlamydia prevalence of the village populations. Two cotton-tipped swabs were used to collect consecutive samples from the left eye. One of the swabs was used for both RNA extraction and chlamydia PCR and was placed immediately into guanidinium isothiocyanate buffer (11) and frozen by using a liquid nitrogen-containing canister. Samples were stored at -70°C in the local hospital laboratory until they could be transported to Johns Hopkins on dry ice. Subjects ranged in age from 1 to 68 years and included 28 males and 22 females. Informed consent was obtained from all individuals in accordance with the guidelines of the Johns Hopkins Committee on Clinical Investigation.

**RNA extraction and cDNA synthesis.** Total RNA was extracted from half of the RNA sample by the guanidinium acid phenol method (11). To facilitate the precipitation of an anticipated small amount of RNA from the human conjunctival samples, glycogen (final concentration, 37%; Boehringer Mannheim, Indi-

anapolis, Ind.) was added at the isopropanol step. cDNA was synthesized by using oligo(dT)<sub>18</sub> (Stratagene, La Jolla, Calif.) and RNase H-negative Moloney murine leukemia virus reverse transcriptase (Stratascript; Stratagene). Conjunctival swab samples from laboratory personnel and tissue cultured conjunctival 1-5C-4 cells (ATCC CCL-20.2; American Type Culture Collection, Rockville, Md.) were used as negative controls. Stimulated mononuclear cells from a healthy volunteer were used as positive controls for PCRs as described in the next section.

**PCR-enzyme immunoassay (EIA).** Semiquantitative reverse transcription-PCR was performed, and products were analyzed by PCR-EIA. Outer primers were used to generate the target DNA (Table 1). Primer sequences were chosen from published sequence information, and the sense and antisense primers were separated by several exons. Reagents for a 100-μl PCR included 0.5 μM primers, 0.2 mM deoxynucleoside triphosphates, PCR buffer (Boehringer Mannheim), and 2.5 U of *Taq* polymerase (United States Biochemical-Life Sciences, Cleveland, Ohio). The thermofile (9600 Thermocycler; Perkin Elmer, Norwalk, Conn.) was set for an 80°C hot start for 4 min, followed by 94, 55, and 72°C for 1, 1, and 2 min, respectively, for 30 cycles and a terminal 10-min extension at 72°C. Different quantities of sample cDNA, including 1, 2, and 4 μl from each sample, were amplified and measured in the EIA to encompass the linear range for this assay.

The specific probe for each target was made from DNA completely nested in the external primers (Table 1). The nested sense primer contained the T7 RNA polymerase promoter sequence at the 5' end, and biotinylated RNA was made in a standard transcription reaction with biotin-11-UTP (Enzo Diagnostics, Farmingdale, N.Y.). Nested DNA for the probes was made from stimulated peripheral blood mononuclear cells (PBMC), and the DNA was cloned into a TA vector (Invitrogen, San Diego, Calif.) and sequenced to verify its identicalness to the published sequence. PBMC were isolated from healthy volunteers by using Ficoll-Hypaque (Pharmacia, Piscataway, N.J.) gradient centrifugation in accordance with the manufacturer's instructions. After 7 days of incubation in RPMI

TABLE 2. Comparison of clinical grade with infection

Clinical grade	No. (% of total)	No. (%) chlamydia positive <sup>a</sup>
TI	19 (38)	12 (63.2)
TF	8 (16)	3 (37.5)
TC	7 (14)	3 (42.8)
TIC	7 (14)	4 (57.1)
NS	9 (18)	2 (22.2)
Total	50 (100)	24 (48)

<sup>a</sup> Chlamydia result positive by PCR.

(Life Technologies, Grand Island, N.Y.) and 10% heat-inactivated human serum, approximately  $2 \times 10^6$  PBMC were either not stimulated or stimulated with either crude phytohemagglutinin (Life Technologies), 1:4,000 soluble tetanus toxoid (Lederle Laboratory), or 2  $\mu$ g of lipopolysaccharide-20 ng of phorbol myristate acetate (Calbiochem, La Jolla, Calif.). Cells were pulsed with 100  $\mu$ Ci of [<sup>3</sup>H]thymidine and incubated for another 24 h. Radioactivity measurements of >1,000 cpm were considered positive. Approximately  $10^6$ -cells-per-milliliter aliquots were frozen at  $-70^\circ\text{C}$  until they could be analyzed.

A microtiter plate method that has been used for other applications in our laboratory was used to detect amplified products (6, 13). Briefly, a biotinylated RNA probe for a respective target is hybridized in solution to the amplified DNA and hybrids are captured in wells of a microtiter plate coated with antibiotin antibody. Bound hybrids are reacted with a monoclonal antibody-alkaline phosphatase conjugate which recognizes the conformation of RNA-DNA in solution (gift from Linda Anderson-Mausser, Miles Laboratory) (8). The substrate, methyl-umbelliferyl phosphate, is fluorogenic when dephosphorylated, and the signal intensity is measured by a fluorometer and expressed as fluorescence units (FU). Signal intensity is proportional to the amount of bound hybrid within the linear range of the assay, which, in our experience, is between 1 and 1,000 gene copies.

The negative cutoff was determined as the mean of five samples of uninfected 1-5C-4 cells plus five standard deviations. To normalize between PCR-EIA runs, this value was subtracted from the test sample value so that values above  $\log_{10}$  0.03 FU would be considered positive. To compare PCR results between conjunctival samples, all samples for a particular cytokine were included on one PCR-EIA run along with amplified dilutions of cDNA from stimulated PBMC corresponding to 10, 100, and 1,000 cells. Since it was not possible to measure the small amount of RNA extracted from a conjunctival swab sample, relative  $\beta$ -actin transcripts were measured from 2, 4, and 5  $\mu$ l of sample cDNA to determine mRNA quality and to obtain a relative measure of quantity in each conjunctival sample. Adjustments for the amount of transcribable mRNA between samples were then made for the cytokine PCRs by amplifying the corresponding volume for each sample which gave an equivalent EIA signal for  $\beta$ -actin in the exponential range of the PCR and the linear range of the EIA. Sample RNA was also amplified with the  $\beta$ -actin primers to check for DNA contamination.

**Processing and PCR for *C. trachomatis*.** The remainder of the guanidium sample was extracted with neutral buffered phenol, and DNA was alcohol precipitated. PCR for a conserved area of the *C. trachomatis* major outer membrane protein gene was performed as previously described (6). As a positive control, dilutions of 2.5 and 2,500 inclusion-forming units of *C. trachomatis* trachoma biovar B (strain Har-36, ATCC VR-573) were detergent-protease lysed, boiled, and amplified.

**Analysis of data.** Because the distributions of fluorescence values from cytokine determinations were not symmetric, they were log transformed. The Wilcoxon rank-sum test was used to test for significance of the difference between median  $\log_{10}$  cytokine values in different infectivity and clinical subgroups. Comparisons among clinical and infectivity subgroups were made by using the chi-square test for contingency tables.

## RESULTS

**Comparison of infection and clinical score.** In this study, we expected to find increased chlamydial burden, as represented by greater chlamydial DNA levels, in association with follicular inflammation or scarring and inflammation. A description of clinical grade by infection status is shown in Table 2. For the subgroup of 50 samples, the proportion positive for chlamydia was 52% for any disease versus 22% for no disease. Mean  $\log_{10}$  FU values  $\pm$  the standard error of the mean for samples that were positive for chlamydial DNA as related to clinical status were as follows: TI,  $2.31 \pm 0.35$ ; TF,  $1.61 \pm 0.18$ ; TC,  $1.41 \pm 0.22$ ; TIC,  $2.02 \pm 0.24$ ; NS,  $0.8 \pm 0.11$ . For comparison, the

ranges for three determinations of dilutions of a stock culture of *C. trachomatis* serovar B were  $1.56 \pm 0.36$  and  $3.26 \pm 0.57$  for 2.5 and 2,500 inclusion-forming units, respectively. Chlamydial load in clinical samples as indicated by FU value was higher when inflammation was present.

**Comparison of infection with cytokine transcripts.** Median transcript levels compared to infection status are shown in Fig. 1. Chlamydia positivity was defined as PCR positivity. IFN- $\gamma$  transcript levels were significantly higher in those who were chlamydia positive than in those who were chlamydia negative ( $P < 0.001$ ). Transforming growth factor  $\beta 1$  (TGF- $\beta 1$ ), IL-2, tumor necrosis factor alpha (TNF- $\alpha$ ), and IL-1 $\beta$  transcripts were also higher in those who were chlamydia positive than in those who were chlamydia negative ( $P < 0.05$ ). Levels of IL-12 p40 transcripts did not differ by infection status.

**Comparison of clinical score with cytokine transcripts.** A comparison of median transcript levels by clinical category is shown in Fig. 2. For the comparison of TI versus NS, TGF- $\beta 1$ , TNF- $\alpha$ , and IFN- $\gamma$  transcript levels were significantly higher in the TI group ( $P < 0.001$ ). For the comparison of TF versus NS, TGF- $\beta 1$ , IFN- $\gamma$ , and TNF- $\alpha$  transcript levels were significantly higher in the TF group ( $P < 0.05$ ). A comparison of any scarring (TC or TIC) versus no disease indicated that TGF- $\beta 1$  transcript levels were elevated ( $P < 0.05$ ). Overall, IL-1 $\beta$  transcript levels were more likely to be elevated in individuals with clinical disease than in those with no disease. Transcripts were also found in seven individuals when signs of disease were not visible, but two of the seven were chlamydia positive. IFN- $\gamma$  transcripts were also found in three individuals with no signs of disease, but chlamydia DNA was detected in all three.

As seen in Fig. 2, IL-2 and IL-12 p40 mRNA levels were both elevated for TI compared with all other disease signs but IL-12 p40 transcripts were also found in some people with TF alone. In addition, IL-12 transcripts were significantly associated with the presence of IFN- $\gamma$  transcripts ( $P < 0.003$ ). Of the 10 individuals who had detectable IL-12 p40, only 6 were chlamydia positive, and this association was not significant, as mentioned previously. However, all 10 individuals were in the TI or TF groups. IL-2 transcripts were more likely to occur in the presence of IL-12 p40 and IFN- $\gamma$  transcripts in a sample ( $P < 0.001$ ). IL-4, IL-5, and IL-10 were not detected in any samples, and IL-6 was detected in only two individuals in association with the TI category (data not shown). CD3- $\delta$  pan

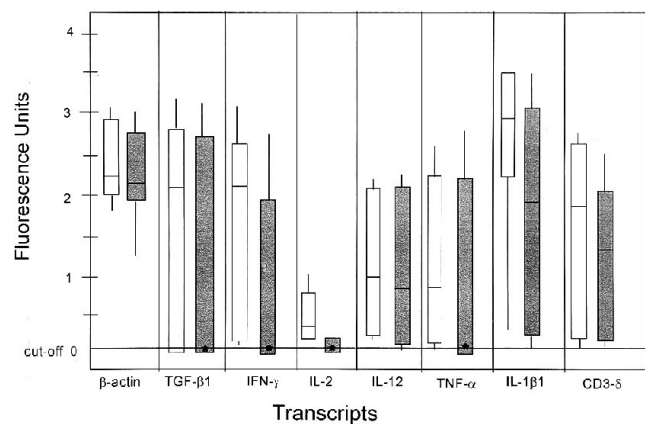


FIG. 1. Transcript levels by infection status. Measurements are in  $\log_{10}$  FU. Boxes encompass 75% of the data. A median falling within a box is indicated by a horizontal line across the box, and a median with an asterisk corresponds to the value at the bottom or lowest point of the box.  $\square$ , chlamydia positive ( $n = 24$ );  $\blacksquare$ , chlamydia negative ( $n = 26$ ).

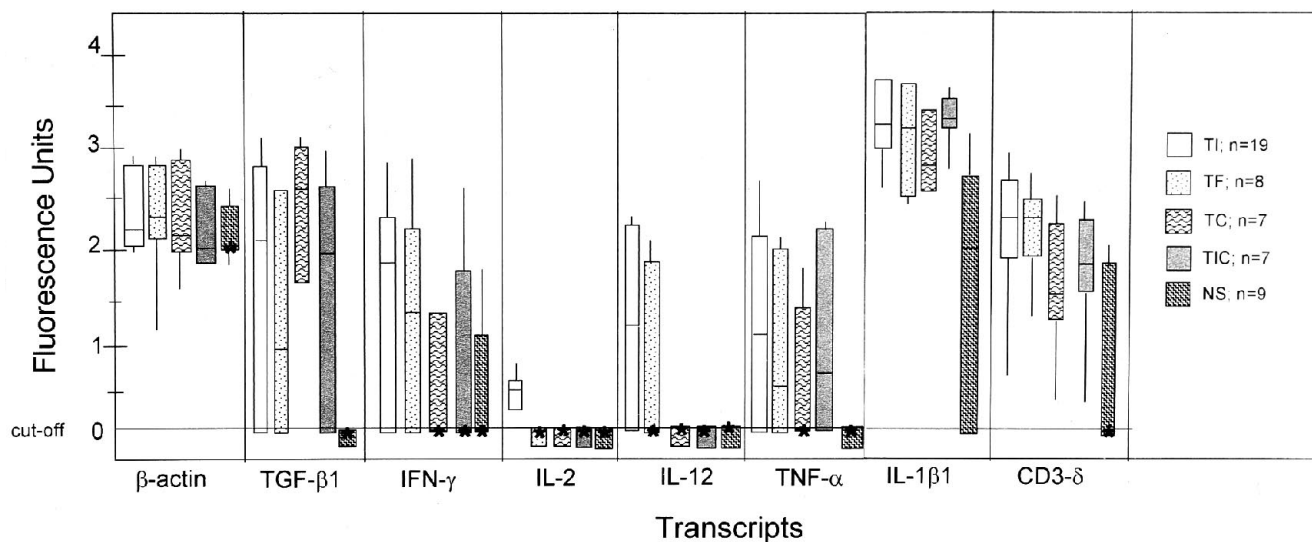


FIG. 2. Transcript type versus clinical status. Clinical categories defined in the text are listed on the x axis. Measurements are in log FU, and 75% of the data reside within the box. A median within a box is represented by a horizontal line across the box, and a median with an asterisk corresponds to the value at the bottom or lowest point of the box.

T-cell receptor transcripts were detected in the conjunctival swab samples in similar amounts when disease was present, but median transcript levels were lower in the absence of disease ( $P < 0.001$ ) (Fig. 2). Cytokine transcripts were not detected in uninfected conjunctival 1-5C-4 cells or conjunctival swab samples of healthy volunteers from an area where trachoma is not endemic.

**Standardization and controls.** A great deal of effort was made to ensure that comparisons of cytokine transcript levels on the basis of PCR-EIA values were made with samples that were approximately equivalent with respect to  $\beta$ -actin cDNA content. Most samples, regardless of disease or infection status, gave similar  $\beta$ -actin PCR-EIA values for either 2 or 4  $\mu$ l of cDNA, indicating that there was relative similarity in template amounts between samples. As seen in Fig. 1, there was no significant difference in median  $\beta$ -actin levels when infected individuals and those with no infection were compared or when the presence of any disease was compared with no disease (Fig. 2). As a control for DNA contamination, RNA was amplified for  $\beta$ -actin and found to be negative for detectable DNA.

DNAs from stimulated PBMC were standardized to the same  $\beta$ -actin values used for clinical samples and served as positive controls for the various conjunctival reverse transcription-PCRs. Although IL-4, IL-5, and IL-10 transcripts were not found in the clinical samples, these transcripts were readily detected from lipopolysaccharide-phorbol myristate acetate- or phytohemagglutinin-stimulated PBMC (FU range, 1.5 to 2), in contrast to unstimulated PBMC (0.04 FU) (data not shown).

## DISCUSSION

In this study, we wished to determine if specific cytokine transcript types or increased levels are a function of severe disease, that is, follicular inflammation and severe scarring, in the presence or absence of *C. trachomatis*. Conversely, we wondered if a particular cytokine pattern in the absence of chlamydia infection and inflammation could represent a more protective response. We have shown that the cytokine response to chlamydial infection is predominantly proinflammatory with respect to IL-1 $\beta$ , TNF- $\alpha$ , IFN- $\gamma$ , and TGF- $\beta$ 1. The level of

chlamydial DNA was higher when inflammation was present, that is, in the TI and TIC groups, and this was in agreement with a past study using PCR for detection of *C. trachomatis* in trachoma (7). In one study, TNF- $\alpha$  response was related to intensity of infection by tissue culture (14). We were not able to directly compare chlamydial DNA load to individual cytokine transcript levels, since chlamydia PCRs were not done as multiplexes with cytokine PCRs. In addition, we were also able to detect chlamydia DNA in the absence of clinical disease (22%). This is consistent with the previous trachoma study (7), in which 22% of individuals with no clinical signs by field grading had chlamydial DNA. Later inspection of conjunctival photographs indicated that 70% of these individuals had between one and four follicles by photograting. Other etiologies which may initially resemble mild trachoma include vernal or allergic conjunctivitis, which causes a self-limited follicular conjunctivitis. In addition, conjunctivitis due to other bacteria is rarely encountered and the clinical picture of purulent discharge is not usually associated with trachoma. We could not say with certainty that low-level or inapparent infection in the absence of disease compared with a certain cytokine pattern constitutes a protective response because of the cross-sectional nature of this study. Overall, cytokine transcript levels were highest when both inflammatory disease and chlamydia were present versus disease and no chlamydia.

In previous studies using monocyte-macrophage lines and mouse infection models, IL-1 and TNF- $\alpha$  were induced by chlamydia infection or chlamydial lipopolysaccharide (29, 38, 48). These cytokines stimulate inflammation by inducing prostaglandins, and both are potent stimulators of tissue remodeling by inducing collagenase production from fibroblasts (16, 17). IL-1 and TNF- $\alpha$  are produced primarily by monocyte-macrophages, but a number of other cell types can produce IL-1, including endothelial cells, epithelial cells, and fibroblasts. Individuals with disease in this study had high IL-1 $\beta$  transcript levels in all disease categories, compared with the low-to-moderate transcript levels in seven people with no signs of disease, two of whom were infected. Laboratory personnel were negative for IL-1 $\beta$  transcripts. Several factors may explain this finding. The study area is very dry, and there is much

particulate matter generated by dust, wood fragments, and smoke. Ocular foreign bodies are a common finding. It is possible that these environmental irritants could represent a constant source of stimulation for IL-1 $\beta$  transcription, which could, in turn, enhance disease severity in some people who experience ocular chlamydia infection. In terms of its biology, IL-1 is a pluripotent and pleiotropic cytokine, and regulation of its ultimate cellular effects occur at several levels. Some controls include the existence of multiple types of response elements in the promoter regions for different transcription factors, the amount of posttranslational processing of pro-IL-1 $\beta$  by IL-1 $\beta$  convertase, and the quantity of IL-1 receptor antagonist in competition with IL-1 $\beta$  for its receptor. In a study measuring IL-1 protein in tears of people with trachoma, low levels of IL-1 were found in those with no disease but levels were much higher when follicular inflammation was present (37).

Elevated median TNF- $\alpha$  levels were most highly associated with the presence of inflammation or follicles, and scarring with inflammation combined with chlamydia positivity. TNF- $\alpha$  production may be translationally regulated (21), but our data still suggest that TNF- $\alpha$  may be a participant in the inflammatory response initiated by chlamydia infection. These results are supported by studies using the mouse pneumonitis model of infection and the guinea pig inclusion conjunctivitis strain of *C. psittaci* in a genital infection model (14, 48). In addition, chlamydial elementary bodies and lipopolysaccharide were able to stimulate TNF- $\alpha$  from blood of healthy volunteers (24). However, it is not clear how TNF- $\alpha$  might contribute to tissue damage or what cells produce it in chlamydia infection. Although macrophages are an important source of TNF- $\alpha$ , our finding of both high IFN- $\gamma$  and TNF- $\alpha$  levels with low IL-2 levels suggests that natural killer cells should also be considered as a potential source of these cytokines during *C. trachomatis* ocular infection.

TGF- $\beta$ 1 transcript levels were elevated in association with all disease manifestations, but especially with scarring (TC category). TGF- $\beta$ 1 is important in wound healing, but constant production can induce pathogenic fibrosis (9). In one study on the modulation of pathogenic scarring in response to trauma, neutralizing antibodies to TGF- $\beta$ 1 were effective in suppressing scar formation without interfering with normal dermal architecture and tensile strength of tissue (41). Our data suggest that TGF- $\beta$ 1 is stimulated during chlamydia infection and could play an important role in progressive scarring in chronic infection.

In terms of the Th1 effectors IL-2 and IL-12 p40, elevated transcript levels were associated with the TI category but not with the later infection sequelae of scarring. Since IL-12 p40 transcripts are tightly associated with bioactive protein (20), a Th1-type response seems to be associated with the initial inflammatory manifestations of trachoma and, to a lesser extent, with follicles. IL-12 also functions in natural killer cell activation and cytotoxic lymphocyte maturation (44, 50). In one study, IL-12 p40 transcripts were found in pleural fluid cells of patients with tuberculous pleuritis, and IL-12 bioactive protein was produced from pleural fluid PBMC in response to *Mycobacterium tuberculosis* (53). IL-12 protein was also found in pleural fluid but not in serum. The investigators suggested that local IL-12 could aid in mycobacterial clearance by triggering IFN- $\gamma$  production by NK and T cells. It would be important to determine if IL-12 could play a similar role in trachoma, since both NK cells and cytotoxic lymphocytes can contribute to protection and lysis of infected target cells in animal models of chlamydia infection (43, 49).

Highest median transcript levels of IFN- $\gamma$ , which can also be

associated with Th1 response, were significantly correlated with chlamydia positivity and with the disease manifestations in the TI and TF categories. In this study, the association of IL-12 p40 transcripts with IFN- $\gamma$  ( $P < 0.003$ ) is consistent with the known up-regulatory effect of IL-12 p40 on IFN- $\gamma$  production by local NK or T cells (27). Some functions of IFN- $\gamma$ , such as induction of both major histocompatibility complex class I and II antigens, activation of macrophages, and enhancement of NK cell activity, have been associated with resolution of some infections. However, in vitro studies suggest that IFN- $\gamma$  may be involved in persistence. This has been demonstrated in one study by using both TNF- $\alpha$  and IFN- $\gamma$  (42). In other studies, a major outer membrane protein-negative, heat shock protein-positive state in chlamydia-infected tissue culture cells in association with aberrant reticulate-body-like forms was induced with IFN- $\gamma$  treatment of cells (5). In our study, some individuals with both inflammatory scarring (TIC) and chlamydia DNA also had IFN- $\gamma$  transcripts. However, three individuals in the NS category had both detectable chlamydial DNA and IFN- $\gamma$  transcripts. It is clear from our study that IFN- $\gamma$  is associated with chlamydia infection, but since our study is cross-sectional, it is not obvious what contribution IFN- $\gamma$  makes to chlamydial clearance and protection from disease. This finding was corroborated in a recent trachoma study, in which IFN- $\gamma$  protein levels were the same in people who were persistently infected and in those who cleared the infection (2).

Cytokines characteristic of a Th2 response, such as IL-4 and IL-10, were not detected in any samples, despite the presence of mononuclear cells as indicated by equivalent amounts of CD3- $\delta$  transcripts associated with disease. Transcripts for IL-6, which, among other effects, provides B-cell help, were found in only two people in the TI category. It has been shown in lung fibroblasts that glucocorticoids can inhibit IL-1-induced IL-6 at both the transcriptional and translational levels (54). It is not known whether this is the case in the conjunctivum. Our findings are partially corroborated by a recent study demonstrating protection from genital chlamydia infection in mice after adoptive transfer of splenic CD4<sup>+</sup> cells (45). Stimulated spleen cells from primary and secondary infections produced high levels of IFN- $\gamma$ , IL-2, and IL-6 but very little IL-4 and no IL-5 protein. Several possibilities could explain the absence of a pro-antibody response characterized by IL-4 and IL-10. Since tear antibodies are found in association with disease in trachoma, antibodies could come from draining lymph nodes. However, another explanation could be that our conjunctival sampling was too superficial and IL-4- or IL-10-producing cells could be found by biopsy of the deeper tissue layers. The presence of mononuclear cells in the samples, as indicated by detectable CD3- $\delta$  transcripts, makes the absence of IL-4- and IL-10-producing T cells due to sample inadequacy less likely. Recently, IL-12 has been shown to induce antigen-specific, complement-fixing immunoglobulin G2a, G2b, and G3 subclass antibodies (18), and IL-12 can potentiate an established Th2 response in humans (25). The possible role of IL-12 in enhancing certain aspects of a Th2 response in immunity to chlamydia should be investigated.

To more clearly define protective and pathologic cytokine responses, we are prospectively studying cohorts of individuals who are exposed to similar societal and environmental risk factors in an area where trachoma is endemic but who have differential responses to infection in terms of chlamydia clearance and disease persistence. It is possible that another trachoma control strategy, in addition to infection control, would be to interfere with disease progression from inflammation to scarring and trichiasis.

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