

Pathway-Focused Arrays Reveal Increased Matrix Metalloproteinase-7 (Matrilysin) Transcription in Trachomatous Trichiasis

Martin J. Holland,^{1,2} David Jeffries,² Michael Pattison,² Gerit Korr,¹ Alevtina Gall,² Hassan Joof,² Ahmed Manjang,² Matthew J. Burton,¹ David C. W. Mabey,¹ and Robin L. Bailey¹

PURPOSE. Several genes that are associated with protection from or susceptibility to trachomatous trichiasis (TT) have been identified through genetic association studies. Yet there have been few studies in which gene expression profiles were assessed in TT cases and disease-free controls. The purpose was to identify genes that are differentially expressed in the upper tarsal conjunctiva of subjects with TT.

METHOD. Pathway-focused gene arrays were used to screen conjunctival RNA expression of 226 gene transcripts of interest. The screening was followed by validation of differentially expressed genes by qRT-PCR on an independent set of samples. Three different techniques were then used to test for quantitative differences in the recovered conjunctival protein fraction.

RESULTS. Focused arrays identified a set of 13 differentially expressed genes. Validation by qRT-PCR confirmed differential expression in four of these genes (*COL1A1*, *COL7A1*, *MMP7*, and *TLR6*). Increased expression of *MMP7* was the only consistent differentially regulated gene in the conjunctival samples of trichiasis subjects. *MMP7* was present in isolated conjunctival proteins and in the tissue culture supernatants of peripheral blood lymphocytes after stimulation.

CONCLUSIONS. There is an imbalance in extracellular matrix turnover with minimal contribution of adaptive immune responses at this stage of trichiasis. There was little evidence of broad differential expression in genes characteristic of polar responses of adaptive T cells or macrophages. The control of the *MMP7* response and its activity appears significant in the fibrotic changes observed in TT. (*Invest Ophthalmol Vis Sci*. 2010;51:3893–3902) DOI:10.1167/iops.09-5054

From the ¹Department of Infectious and Tropical Diseases, London School of Hygiene and Tropical Medicine, London, United Kingdom; and the ²Medical Research Council Laboratories, Fajara, Banjul, The Gambia.

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Corresponding author: Martin J. Holland, Department of Infectious and Tropical Diseases, London School of Hygiene and Tropical Medicine, London WC1E 7HT, UK; martin.holland@lshtm.ac.uk.

Trachoma, a disease caused by infection with *Chlamydia trachomatis*, is now predominantly found in the developing world. Recurrent infection with this bacterium leads to inflammation of the upper tarsal conjunctiva and eventually to scarring. Progressive scarring over time leads, in some individuals, to trachomatous trichiasis (TT), a morphologic change in the eyelid causing the lashes to rub against the globe. This process, which often occurs in the absence of detectable *C. trachomatis* infection, can result in corneal opacity and blindness.¹ An estimated 40 million currently have active trachoma. Of those, 8.2 million have TT, and 1.3 million are irreversibly blind as a result.²

The SAFE strategy is recommended by the World Health Organization (WHO) for the control of blinding trachoma: surgery for trichiasis, antibiotics for infection, facial cleanliness, and environmental improvements to reduce transmission of infection. However, since fibrosis may continue to progress in the absence of current *C. trachomatis* infection, new cases of TT are likely to be seen in endemic communities after transmission of *C. trachomatis* has been controlled. Even after successful surgery, recurrence rates of up to 60% may be seen within 3 years.³ It is therefore important to understand the processes involved in the pathogenesis of TT by gaining a more complete understanding of the tissue-specific responses associated with the disease process.

It is well established that T helper type 1 (Th1) cells are associated with clearance of chlamydial infection.^{4,5} In particular, IFN γ plays an important role in the clearance of chlamydial infection in both mice and humans.^{6,7} However, the inflammatory response, to which IFN γ contributes, may also be the cause of disease if it is excessive or uncontrolled. Normally, the inflammatory response is counterbalanced by IL-10.^{8,9} More recently, regulatory T cells (Tregs) have been identified as important counterinflammatory mediators of disease in several chronic infections, especially at the site of infection.^{10,11} Tregs, which can be identified in the conjunctiva during ocular viral infection, may also play a part in the pathogenesis of trachoma.^{12,13} Additional counterbalancing mechanisms include the evolution of Th2 or type-2 responses. Type 2 responses are frequently associated with chronic inflammation and infection. Although there is some evidence that Th2 responses are contributory to chlamydia's effects,^{6,8,14} there is little convincing evidence that polar Th2 cell responses directly cause fibrosis.

On the other hand, the role of innate responses from epithelia and leukocytes are increasingly recognized as playing an important role in the pathologic inflammatory process. In particular, IL1 β and -8 have both been identified in vitro (from *C. trachomatis*-infected cells¹⁵) and in vivo (from trichiasis patients with inflammation^{16,17}), leading to the suggestion that these are critical factors in the development of inflammatory

disease. There is now a much improved understanding of the interaction of innate responses, autoinflammatory cascades, and tissue homeostasis. In particular, the interaction of the matrix metalloprotease (MMP) family of proteins, proinflammatory cytokines, and innate antimicrobial peptides produced by T cells, NK cells, and macrophages.

Thus far, the investigation of the cellular responses and ECM composition of the conjunctiva or the tear film constituents of patients with trichiasis is limited to a few immunohistochemical and expression studies.¹⁷⁻²¹ Therefore, we investigated whether the expression of other genes involved in innate, adaptive, and extracellular matrix regulation were altered in conjunctival tissue from participants with TT. We show that at this stage of disease, in the almost complete absence of current chlamydial infection, there is little evidence of the involvement of factors produced by the specific adaptive immune response. The profile of responses is characterized by a dominance of gene expression of innate defense molecules and ECM components. In particular, the altered expression of *MMP7* appears strongly associated with fibrotic disease.

METHODS

Study Participants and Samples Collected

Informed consent was obtained from all study participants. The participants were recruited from rural and semi-urban areas within the Western and Lower River Regions of The Gambia. Trachoma was graded by a single experienced field supervisor according to the World Health Organization (WHO) simplified grading system. Subjects with TT (more than one eyelash touching the globe) were identified. For each TT case, an age-, sex-, and location-matched control subject without any signs of conjunctival scarring and who was not a member of the same family was also recruited. Participants were age matched within 5 years of one another. In a standardized manner, an ocular swab from the everted tarsal conjunctiva of each participant was collected into RNA stabilizer (RNAlater; Ambion Europe, Ltd., Huntingdon, UK) for the isolation of proteins and nucleic acids. Venous blood samples were obtained from a subgroup of these subjects. The study was conducted in accordance with the tenets of the Declaration of Helsinki. It was approved in The Gambia by the joint Ethics Committee of the Gambian government and the U.K. Medical Research Council and by the ethics committee of the London School of Hygiene and Tropical Medicine.

Isolation of DNA, Total RNA, and Total Protein from Ocular Swabs

Total RNA was isolated (RNeasy micro kits; Qiagen, Ltd., Crawley, UK) and used as a template to generate probes for miniarrays (SuperArray; Qiagen, Ltd.). Total RNA was eluted in 30 μ L of RNase-free Tris. The total yield and purity of nucleic acid in Tris was estimated by spectrophotometer (NanoDrop ND-1000; Thermo Scientific, Wilmington, DE). RNA samples with low purity (260:280 < 1.8) or with evidence of impurities from the extraction procedure (230:260 > 1.8) were re-extracted by repetition of the extraction protocol with the addition of a twofold increase in wash buffers. These were then reassessed for purity and quantity. Only samples that had a spectrographic profile consistent with those outlined at the Genome Center Maastricht (RNA quality control: NanoDrop ND-1000; Thermo Scientific; http://biomedicalgenomics.org/RNA_quality_control.html) were used for the production of biotin UTP labeled-aRNA. Samples from 11 cases and controls with at least 100 ng total RNA were used as templates to generate aRNA. Assessment of the quality of RNA by microcapillary gels was not available. All subsequent ocular swab samples were extracted (All-Prep Kit; Qiagen, Ltd.), according to the manufacturer's instructions. To obtain maximum yields of DNA, RNA and protein from the ocular swabs, a modified initial step was used. Swabs were transferred to a 2-mL screw-cap tube containing sample lysis buffer. They

were then vortexed vigorously and the swab discarded. The lysate was passed through a column that binds DNA. The column was washed, and the DNA was eluted in 100 μ L of RNase-free water. Ethanol was added to the flow-through from the DNA column and added to a minispin column (RNeasy; Qiagen, Ltd.). Total RNA bound to the membrane was subjected to DNase I digestion. The total RNA was eluted in 60 μ L of RNase-free water. Proteins were precipitated from the flow-through with buffer APP. The precipitated proteins were collected by centrifugation, and total protein was redissolved in 100 μ L of 5% sodium dodecyl sulfate (SDS). DNA and protein samples were stored at -20°C and RNA at -70°C .

Quantification of Total Amounts of Nucleic Acid and Protein

Total RNA was quantified in a 2- μ L sample by reading the absorbance at 260 nm (NanoDrop model ND-1000; Thermo Scientific). Total DNA was quantified by measuring the amount of mitochondrial DNA present in the sample using SYBR-green qPCR. Total protein was quantified in 10- μ L samples with an enhanced bicinchoninic acid (BCA) assay (Pierce Chemical, Rockford, IL) on the spectrophotometer (NanoDrop ND-1000; Thermo Scientific).

Generation of Labeled aRNA Probes and Gene Array Analyses

Total RNA was amplified and labeled with biotin-UTP (Message Amp II kit; Ambion Europe Ltd.). Biotin-labeled aRNA was produced after two overnight rounds of in vitro transcription. Labeled aRNA was hybridized overnight to each membrane and two separate membranes (arrays) were used for each subject (with the exception of one sample from an affected case on one Th1/Th2/Th3 array). All arrays, hybridization solutions, wash, and probe detection kits were purchased (SuperArray Bioscience Corp., Frederick, MD). Each array consisted of 113 genes (two blanks, three artificial sequences, one bacterial plasmid sequence [PUC18], two biotin-coupled positive controls, and five reference genes covering seven positions) that were selected for involvement in pathways focused on human extracellular matrix and adhesion molecules (OHS-013) or human Th1/Th2/Th3 cells (OHS-034). Bound aRNA was detected by the addition of streptavidin-alkaline phosphatase (CDP-Star; Roche Diagnostics, Indianapolis, IN). Images of the membranes were captured with a chemiluminescence detection system (Chemi-Doc; Bio-Rad Laboratories, Ltd., Hemel Hempstead, UK) with a CCD camera after 100 seconds of exposure for ECM arrays and 200 seconds for Th1/Th2/Th3 arrays. Each 16-bit TIFF file was then downloaded into array-analysis software (GEArray Expression Analysis Suite 2.0; SuperArray) for image processing and array analysis. Local background correction for spot intensity was applied to each membrane and the raw spot intensities values were then exported to a spreadsheet along with all array meta-information (Excel; Microsoft, Redmond, WA). The raw spot intensities, images, and array information were then analyzed by using the image-processing and array tool features in commercial software (MatLab; The MathWorks, Natick, MA).

Array Normalization

We tested 11 different array normalization strategies, the most appropriate array normalization method was selected by testing each normalization with the technique described by Kroll and Wolff.²² Normalization of the arrays with the global mean was selected as the best fitting normalization method. The filtered gene list of significantly differentially regulated gene transcripts was then produced based on rank differences in signal intensities. A distribution-free test of significance based on rank difference was used. Genes with rank differences which were significant at the 10% level, without adjustment of *P*-values for multiple testing, were considered differentially expressed. Only those in which at least five subjects had above-background expression values were selected for further analysis. Since multiple array normalization strategies were tested, only genes that appeared in global mean

normalization and at least one of the other 10 normalization strategies tested were selected. Reproducibility and consistency between arrays was tested with a rank-based ANOVA. Last, a categorical nonquantitative analysis, based on presence or absence scoring, was also performed. These categorical data were analyzed by Fisher's exact test, and the results used to complement the quantitative analysis of differential gene expression. We calculated the percentage of false-positive, differentially expressed genes at the 10% and 5% level according to the method described by Stekel.²⁵

Quantitative RT-qPCR

The PCR primer sequences used in this study are listed in Supplementary Table S1 (all Supplementary Tables are available at <http://www.iovs.org/cgi/content/full/51/8/3893/DC1>). qRT-PCR was performed using a two-step protocol previously described.²⁴ Gene expression was quantified in duplicate by real-time qPCR (QuantiTect SYBR Green PCR kit; Qiagen, Ltd.) on a real-time thermal cycler (Rotor-Gene 6000; Corbett Research, Cambridge, UK). Standard curves were used to quantify the copy number in unknown or test samples, as described by Burton et al.²⁰

Mitochondrial DNA qPCR

PCR was performed on genomic DNA with primers for human-specific hypervariable 1 (HV1) D-loop region mitochondrial DNA in conditions described by Harding-Esch et al.²⁵ The amount of DNA in the sample was then expressed as follows: The sample with the highest cycle threshold (Ct_1) was selected as the reference, and all other samples (n) were then estimated relative to this value by multiplying it by two to the power of the difference in the cycle threshold [$Ct_1 * 2(Ct_1 - Ct_n)$].

Testing for *C. trachomatis*

A commercial assay (Amplicor CT/NG; Roche Diagnostics) was used for detection of *C. trachomatis* in cases and controls, as described elsewhere.²⁶ The reaction buffer conditions required were attained by first diluting purified DNA (9 μ L) in 94.5 μ L of a 50:50 mixture of lysis and diluent buffers; 50 μ L was then used in the standard assay. Positive and negative samples were assigned according to the manufacturer's instructions.

SDS-PAGE, Silver Staining, and Immunoblot Analysis

Total protein was pooled from each of the cases and controls, and 10 μ g of protein was denatured by boiling in SDS-PAGE gel-loading buffer and loaded onto 5% to 15% polyacrylamide gradient gels (Bio-Rad Laboratories, Ltd.). Ten microliters of protein-marker IV (peqGOLD; Peqlab, Erlangen, Germany) was loaded as size markers. Total protein in SDS-PAGE gels was visualized by staining (ProteoSilver kit; Sigma-Aldrich, Poole, UK), per the manufacturers' protocol. Immunoblots were prepared as just described after the transfer of proteins from the SDS-PAGE gel to nitrocellulose membranes. The membranes were then preblocked in a TBS-T (Tris-buffered saline, 0.1% Tween) supplemented with 5% powdered milk solution overnight at 4°C before immunoblot analysis. The membranes were incubated with a primary anti-MMP7 antibody (MAB9071; 1:100; R&D Systems Europe Ltd, Abingdon, UK), followed by a secondary anti-mouse IgG horseradish peroxidase-conjugated antibody. Bound antibodies were detected with enhanced chemiluminescence (ECL Plus; GE Health Care, Amersham, UK), according to the manufacturer's instructions. Chemiluminescent output was then visualized with a 1-minute exposure using standard x-ray film. The films were photographed and scanned by a gel documentation system and analyzed (Gel-doc with Quantity-one software; Bio-Rad Laboratories, Ltd.).

In Vitro Culture and Stimulation of PBMCs

PBMCs were isolated and cultured as described elsewhere.²⁷ The cells were co-incubated with 5 μ g/mL of pokeweed mitogen (PWM), sero-

var A *C. trachomatis* elementary bodies (EBs), or culture medium alone. The cells were cultured for 7 days. On day 6, 100 μ L of culture supernatant was removed from each well, followed by the addition of 1 μ Ci/well [³H]thymidine for the last 18 hours of culture, to determine the proliferative index. The culture supernatant was frozen at -20°C until tested for cytokines by capture ELISA.

MMP7 Capture ELISA

MMP7 ELISA kits (Quantikine; R&D Systems) were used to measure amounts of pro- and active MMP7 according to the manufacturer's instructions for tissue culture supernatants. PBMC culture supernatants from 30 cases and controls stimulated for 6 days with pokeweed mitogen, *C. trachomatis* A EBs, or culture medium alone were tested. The quantity of MMP7 in nanograms per milliliter was then estimated from a standard curve performed at the time of the assay for each plate. The sensitivity of the assay was 0.094 ng/mL.

Statistical Analysis

For qRT-PCR and ELISA, a matched-pair analysis was performed using the Wilcoxon signed-rank test. For assays below sensitivity, the data were reclassified as positive or negative, and the pairing was maintained by using McNemar's χ^2 test. We did not estimate the power of the study since, in the absence of any previous studies, it was not possible to estimate the size of the expected differences or the number of genes in a particular pathway that might be affected. Unadjusted *P*-values after multiple testing are presented with the significance level expected after Bonferroni correction.²⁸

RESULTS

Study Group and Sample Characteristics

Table 1 describes patient and sample characteristics of the individuals participating in the different parts of the study. Eleven case-control pairs were studied with gene microarrays (SuperArray), and a further 47 case-control pairs were studied with qRT-PCR. Cases and controls were matched on age, sex, location, and ethnicity, which act as a proxy control for infection exposure history but without the development of scarring disease. Only one case tested positive for *C. trachomatis* infection. Nucleic acid and proteins were isolated from the swabs of these subjects and the levels estimated by using the different assays. The levels of each of these fractions were not significantly different between the cases and controls and indicated no inherent fundamental differences or technical inconsistencies that might later affect measurements of differential gene expression. The total number of genes expressed on each array by each group of cases or controls was extracted from the arrays. No large-scale or significant differences were indicated in the total number of genes expressed between the cases and controls. This lack of significance suggested that the comparison of healthy to diseased tissue expression was appropriate and that altered expression levels of genes normally expressed in the conjunctiva, rather than aberrant gene expression, contribute to the pathologic changes observed.

Genes Involved in ECM Breakdown, Remodeling, and Adhesion

The conjunctival RNA samples of 22 subjects in total, representing 11 case-control, pair-matched samples, were tested on both the ECM and adhesion molecules array and on the Th1/Th2/Th3 array. There was a sufficient yield of aRNA from one sample after amplification to probe only the ECM array. The array platform layout and differential gene expression across the sum of these arrays is visually represented in Figure 1. Genes that were significantly differentially expressed from both array types are shown in Table 2, including accession

TABLE 1. Participant Characteristics for Each Part of the Study

	Cases	Controls
Gene Array Samples		
Age (range), y	67 (49–80)	65 (44–80)
Sex		
Male	3	3
Female	8	8
Total RNA yield (ng/ μ L)	18.72 (8.4–63)	15.8 (10.2–64.4)
CT/NG Amplificor	NT	NT
Median number (range) of genes expressed per array type		
ECM	40 (29–64)	49 (36–65)
Th1/Th2/Th3	33 (19–55)	48 (21–63)
qRT-PCR Samples		
Age (range), y	54 (3–76)	50 (3–75)
Sex		
Male	22	22
Female	25	25
Total RNA yield (ng/ μ L)	13.75 (6.4–29.2)	13.64 (2.9–22.8)
Total DNA* (qPCR)	3300 (67–13790)	3536 (27–39840)
Total protein (ng/ μ L)	309.8 (100–794)	331.4 (119–791)
CT/NG Amplificor (<i>n</i> positive)	1	0

n = 11 for both cases and controls for Gene Array; *n* = 47 for both groups for qRT-PCR. Data are expressed as median (range). NT, not tested.

* Below measurable range (<2 ng/ μ L) of the spectrometer (OD 280 nm) and therefore estimated by relative quantification real-time PCR.

numbers and brief descriptions of gene product function. In total, nine genes were considered significantly upregulated, and three were downregulated in the TT cases compared with the controls. One additional gene (*COL1A1*) was identified as significantly more frequently expressed in the TT cases than in the controls based on categorical scoring of spot presence or absence (Supplementary Table S2). The signal intensities of these spots are close to background. At $P < 0.1$ or < 0.05 , the number of potential false-positive genes with differential gene expression was calculated as 24 and 0.6, respectively. The raw acquired images of the developed arrays are shown in Supplementary Figure S1 <http://www.iovs.org/cgi/content/full/51/8/3893/DC1>, and tables of the raw and background spot intensity values are given in Supplementary Table S3. There were no significant differences between the cases and controls in the total number of genes expressed on each type of array (reference genes and control sequences were excluded). A simple summary table of the genes expressed in the extracted conjunctival RNA is shown in Supplementary Table S4.

Validation of Differentially Expressed Genes in Total RNA from Ocular Swabs from an Independent Set of TT Subjects and Controls

Initially, human ribosomal protein 1 (*HuPO1*), used in several other studies as a housekeeping gene,^{29,30} was selected as a reference gene for qRT-PCR normalization. However, the *HuPO1* copy number per nanogram total RNA was not equal in cases and controls (data not shown) and so was disregarded. As an alternative normalization method, the amount of total RNA or DNA, which was not significantly different between cases and controls, was selected. Normalization by either of these latter measures was considered suitable, since these had minimal effects on the correlation structure of the data and have been used by others.^{31,32} Real-time quantitative PCR was then used in a larger set of independent samples to determine whether the 14 genes identified from the array screening would be differentially expressed when assayed with nonamplified total RNA as the starting template. Assays with sufficient

specificity and sensitivity for *COL19A1* were not developed. Three additional genes, *ARG1*, *CCL18*, and *NOS2*, characteristic of M1/M2 macrophage polarization, were screened at this stage (Fig. 2). Four genes were significantly differentially expressed ($P < 0.05$). *MMP7* was upregulated and consistent with the array results, whereas *COL1A1*, *COL7A1*, and *TLR6* were all found to be downregulated in cases by qRT-PCR and were inconsistent with array results. The assays of four genes (*VCAN*, *LAMB1*, *SOC31*, and *NFATC1*) were not sensitive enough to provide sufficient quantitative data, and the results were therefore reclassified categorically as responder or non-responder and analyzed by McNemar's χ^2 test. None of the four genes was significantly differentially expressed between groups when analyzed in this way. Most of the genes identified from the array and tested by qRT-PCR ($n = 7/12$) were consistent in their expression. *MMP7* was the only gene that was consistent in its significant differential expression by array and by qRT-PCR and was frequently identified as differentially expressed, irrespective of the array normalization method selected. We therefore focused on *MMP7* expression in the recovered conjunctival protein fraction and on its production by in vitro cultured PBMCs stimulated with chlamydial antigens from the cases and controls.

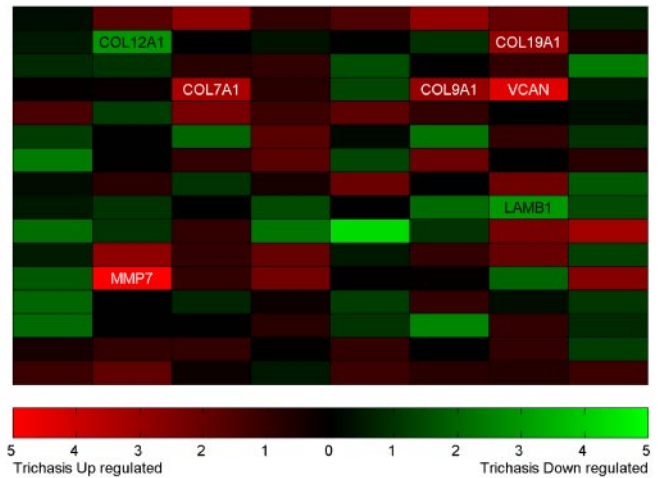
SDS-PAGE Resolution of Isolated Conjunctival Proteins

Silver staining was used to visualize the total protein content recovered from the conjunctival swabs of the cases and controls in SDS-PAGE gels (Fig. 3). The proteins were pooled from each of the 47 cases and controls, and equivalent amounts of protein were loaded on the gel. No gross differences in the number or the intensity of the protein bands were evident. Overall, the cases and controls appeared to have nine major bands. A dominant, intense band at ~60 kDa was present in both groups. The gels were analyzed (Quantity-one software; Bio-Rad) and produced a density profile that suggested a small change between the cases and controls in abundance of proteins below the major band at ~60 kDa.

a

RPS27A	ATF2	BCL3	CCL11	CCL15	CCL5	CCL7	CCR2
CCR3	CCR4	CCR5	CCR9	CD2	CD28	CD4	CD69
CD80	CD86	CEBPB	CREBBP	CSF2	CTLA4	CXCR3	FADD
NFATC2IP	FOS	FOSL1	FOSL2	GATA3	GFI1	GLMN	GPR44
HAVCR2	ICOS	IFNG	IGSF6	IL10	IL12B	IL12RB2	IL13
IL13RA1	IL13RA2	IL15	IL17F	IL18	IL18BP	IL18R1	IL1R1
IL1R2	IL2	IL27	IL2RA	IL4	IL4R	IL5	IL6
IL6R	IL7	IL9	INHA	INHBA	IRF1	IRF4	JAK1
JAK2	JAK3	JUN	JUNB	JUND	LAG3	LAT	MAF
MAP2K7	MAPK10	MAPK8	MAPK9	CIITA	NFATC1	NFATC2	NFATC3
NFATC4	NFKB1	PTPRC	PCGF2	SFTPD	SOCS1	SOCS2	SOCS3
SOCS4	SOCS5	SOCS6	SOCS7	SPP1	STAT1	STAT4	STAT6
TBX21	TFCP2	TGFB3	TH1L	TLR4	TLR6	TMED1	TNF
TNFRSF21	CD27	TNFRSF8	TNFRSF9	TNFSF4	CD40LG	FASLG	TYK2
YY1	ZFPM2	PUC18	Blank	Blank	AS1R2	AS1R1	AS1
GAPDH	B2M	HSP90AB1	HSP90AB1	ACTB	ACTB	BAS2C	BAS2C

b



c

RPS27A	ADAMTS1	ADAMTS13	ADAMTS8	CD44	CDH1	CNTN1	COL11A1
COL11A2	COL12A1	COL14A1	COL15A1	COL16A1	COL18A1	COL19A1	COL1A1
COL24A1	COL27A1	COL4A2	COL4A3	COL4A6	COL5A1	COL5A3	COL6A1
COL6A2	COL6A3	COL7A1	COL8A1	COL8A2	COL9A1	VCAN	CTGF
CTNNA1	CTNNA1	CTNND1	CTNND2	ECM1	FN1	HAS1	ICAM1
ITGA1	ITGA10	ITGA11	ITGA2	ITGA2B	ITGA3	ITGA4	ITGA5
ITGA6	ITGA7	ITGA8	ITGA9	ITGAL	ITGAM	ITGAV	ITGAX
ITGB1	ITGB2	ITGB3	ITGB4	ITGB5	ITGB6	ITGB7	ITGB8
KAL1	LAMA1	LAMA2	LAMA3	LAMA4	LAMA5	LAMB1	LAMB2
LAMB3	LAMB4	LAMC1	MMP1	MMP10	MMP11	MMP12	MMP13
MMP14	MMP15	MMP16	MMP17	MMP2	MMP20	MMP24	MMP26
MMP3	MMP7	MMP8	MMP9	NCAM1	PECAM1	SELE	SELL
SELP	SGCE	SPARC	SPG7	SPOCK1	SPP1	TGFBI	THBS1
THBS2	THBS3	THBS4	TIMP1	TIMP2	TIMP3	CLEC3B	TNC
VCAM1	VTN	PUC18	Blank	Blank	AS1R2	AS1R1	AS1
GAPDH	B2M	HSP90AB1	HSP90AB1	ACTB	ACTB	BAS2C	BAS2C

d

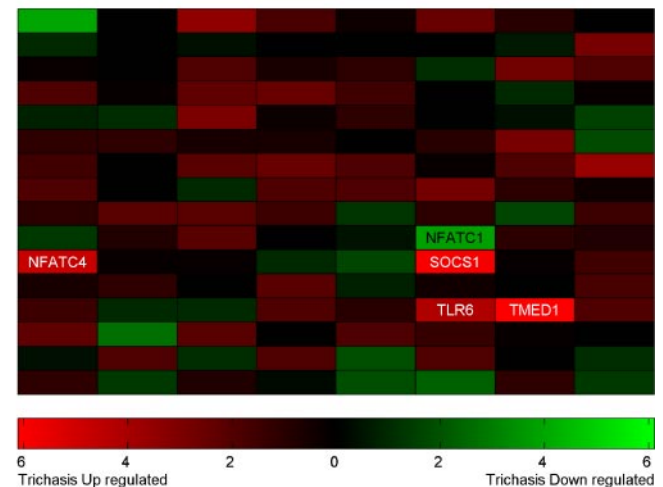


FIGURE 1. Total RNA was isolated from conjunctival swabs of 11 trichiasis cases and 11 age-, sex-, and location-matched controls. mRNA was amplified, biotin labeled, and used as a probe on each array. The array layouts with gene symbols are shown in (a) for ECM and cell adhesion molecules and in (c) for Th1/Th2/Th3. (b, d) Genes in which the rank difference was significant at the 10% level are indicated by their designated gene symbol. (Red) Genes with increasing intensity of expression; (green) decreasing intensity of expression between cases and controls for each array type.

Detection of MMP7 in Recovered Conjunctival Proteins by Immunoblot Analysis

The molecular weight of pro-MMP7 is 28 kDa; however, the recombinant hMMP7 (MMP7 Quantikine kit; R&D Systems) is produced in carrier form with a larger molecular weight. Figure 4, lane 2, shows a large band at ~70 kDa when probed with anti-MMP7 that served as a positive control reaction. When probed with secondary antibody alone (lane 3) there was no reactivity. A band of equal intensity was visible in lanes 4 (pooled cases) and 6 (pooled controls) at ~26 kDa, when probed with monoclonal anti-MMP7 antibody. No bands were visible in lanes 5 (pooled cases) and 7 (pooled controls) when probed with secondary antibody alone.

Quantification of MMP7 by Quantikine Capture ELISA

Since quantitative differences between the cases and controls could not be determined with immunoblot analysis, we attempted to measure MMP7 levels in the individual samples of

recovered conjunctival proteins and in PBMC tissue culture supernatants. PBMCs were used as a source of cells that express abundant levels of MMP7, to complement the estimates of MMP7 levels directly recovered from the conjunctival proteins. However, the levels of MMP7 could not be measured in recovered total conjunctival protein collected from ocular swabs because of the inhibition of the assay by the samples. MMP7-spiked samples remained inhibited despite dilution of the samples and exchange of sample in appropriate buffers and diluents. MMP7 levels in culture supernatant of PBMCs appear to accumulate over time since at 2 days after culture, levels were not detectable (data not shown). By 6 days of culture spontaneous production of MMP7 in unstimulated cultures was highest in both the cases and controls, although highly variable (Fig. 5). Stimulation with either PWM or EBs inhibited or blocked the spontaneous production of MMP7 by PBMCs. This difference in levels reached borderline significance in response to stimulation with EBs, such that in the cases, stimulation with EBs had a smaller effect on the inhibition of MMP7 production than in the controls. The degree of inhibition or the amount of

TABLE 2. Differentially Expressed Genes Identified from ECM and Th1/2/3 Arrays

Gene Symbol	Accession No.*	Gene Name (Function)	Change Ratio†	Rank Difference	P
Extra Cellular Matrix and Adhesion Molecules					
Upregulated					
<i>COL7A1</i>	NM_000094	Collagen, type VII, alpha 1 (Adhesion molecule, basement membrane constituent, ECM structural constituent, ECM protease inhibitor)	0.94	+3.36	0.0981
<i>COL9A1</i>	NM_001895	Collagen, type IX, alpha 1 (Adhesion molecule, ECM structural constituent)	1.34	+3.00	0.0695
<i>COL19A1</i>	NM_001858	Collagen, type XIX, alpha 1 (Cell-cell adhesion, ECM structural constituent)	0.99	+3.00	0.0695
<i>MMP7</i>	NM_002423	Matrix metalloproteinase 7 (ECM protease, epithelial cell inflammatory response to virulent bacteria, activation of α -defensins and other innate factors)	4.30	+5.00	0.0665
<i>VCAN</i>	NM_004385	Versican (Basement membrane connective tissue link protein, tumour metastasis)	1.30	+4.45	0.0404
Downregulated					
<i>COL12A1</i>	NM_004370	Collagen, type XII, alpha 1 (Adhesion molecule, ECM structural constituent)	0.53	-3.00	0.0695
<i>LAMB1</i>	NM_002291	Laminin, beta 1 (Structural basement membrane protein)	0.79	-3.00	0.0695
Th1/Th2/Th3					
Upregulated					
<i>NFATC4</i>	NM_004554	Nuclear factor of activated T cells, cytoplasmic, calcineurin dependent-4 (Th2 and induction of IL4 and IL2)	1.84	+4.77	0.0783
<i>SOCS1</i>	NM_003745	Suppressor of Cytokine signaling 1 (Th1 cytokine induced inhibition of IFN γ signaling)	1.54	+5.92	0.0262
<i>TLR6</i>	NM_006068	Toll-like receptor 6 (innate inflammatory response to bacterial lipoproteins with TLR2)	1.34	+4.20	0.0763
<i>TMED1</i>	NM_006858	Transmembrane emp24 protein transport domain containing 1 (Th2, NK2 and NKT2 surface-expressed marker)	2.36	+6.11	0.0242
Downregulated					
<i>NFATC1</i>	NM_172390	Nuclear factor of activated T cells, cytoplasmic, calcineurin dependent-1 (Th2 cytokine gene expression)	0.37	-3.92	0.0399

* <http://www.ncbi.nlm.nih.gov/Genbank>; provided in the public domain by the National Center for Biotechnology Information, Bethesda, MD.

† Change ratio of expression derived from raw non-normalized values from the array. Percentage of false positives at $P < 0.05 = 15$; $P < 0.1 = 100$.

MMP7 production did not correlate (positively or negatively) with either the PBMC proliferative index or production of IFN γ (data not shown).

DISCUSSION

We investigated conjunctival gene expression in subjects with TT using low-density, focused gene expression arrays and qRT-

PCR, targeting the genes involved in inflammation and ECM composition. Validation of focused array data by RT-PCR confirmed the differential expression of genes involved in ECM regulation (*MMP7*, *COL1*, and *COL7*) and innate immune responses (*TLR6*), but only *MMP7* expression was consistent between results obtained with arrays and qRT-PCR. There was no evidence from qRT-PCR of differential regulation of Th2 transcription factors, NK2 cell markers, or suppression of IFN γ signaling by *SOCS1*. The addition of *CCL18*, *NOS*, and *ARG* to

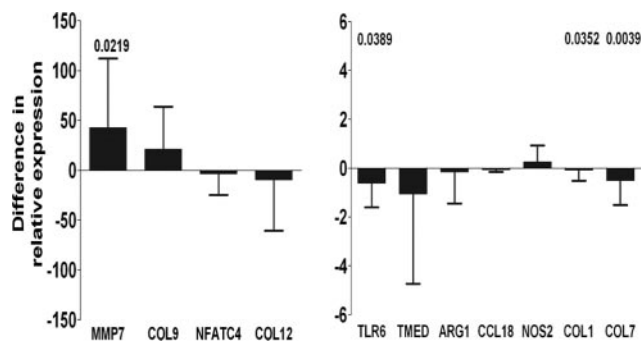


FIGURE 2. Median difference (with upper or lower quartile range) in relative expression from total RNA conjunctival samples of 47 cases and 47 controls measured by qRT-PCR with primers specific for each gene. Positive differences indicate an upregulation in disease and negative differences indicate a reduction in expression. Unadjusted significant P -values ($P < 0.05$), shown above each target gene, were calculated with Wilcoxon-signed rank test. Bars indicate significantly downregulated genes. Bonferroni correction for multiple testing estimates significant values needed to reach 0.05/16 ($P < 0.003125$).

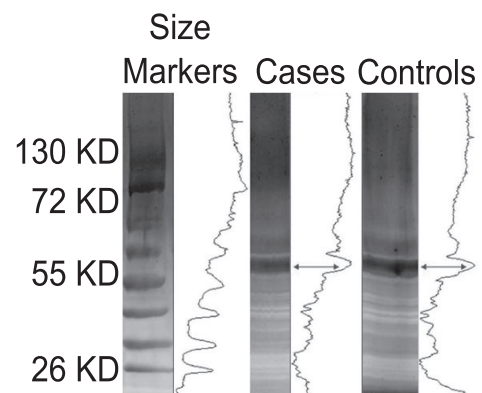


FIGURE 3. Conjunctival protein fractionation from pooled cases (10 μ g) and pooled controls (10 μ g) run on 5% and 15% SDS-PAGE silver-stained gels. Density profiles are shown to the right of each lane, indicating a small change in profile shape and height below the major 60-kDa band (arrows).

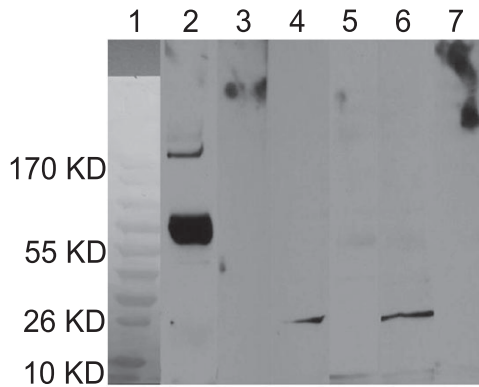


FIGURE 4. Immunoblot using conjunctival proteins recovered from cases and controls. Total pooled proteins (10 μ g) were transferred to nitrocellulose membranes and probed with an anti-MMP7 primary (1^o) antibody. Bound antibody was detected with a secondary (2^o) antibody conjugated to HRP (anti-mouse IgG-HRP) and detected by ECL. Control lanes were probed with 2^o antibody alone. Molecular weight markers were used in *lane 1* to estimate the size of unknown bands from pooled samples. *Lanes 2* and *3* were loaded with 0.1 ng of recombinant human MMP7 (rhMMP7) as a positive control (~70 kDa when probed with anti-MMP7). Probing with 2^o antibody alone (*lane 3*) elicited no reactivity. A band at ~26 kDa was visible in *lanes 4* (pooled cases) and *6* (pooled controls) when probed with monoclonal anti-MMP7 antibody. No bands were visible in *lanes 5* (pooled cases) and *7* (pooled controls) when probed with 2^o antibody alone.

screen for macrophage polarization found no evidence of polarization.

MMP7 upregulation is widely found in other fibrotic diseases and tumor metastasis.^{33,34} MMP7 is naturally expressed in epithelia and injured tissue³⁵ and is increased in alternatively activated (M2) macrophages.^{36,37} MMP7 has a wider role than ECM regulation, with an increasingly recognized position in innate defense by its action on latent TNF, α -defensins, and FAS ligand.^{38–40} Mice deficient in MMP7 (*mmp7*^{-/-}) that lack functional intestinal α -defensins are highly susceptible to infection by enteric pathogens. They also have reduced levels of active FAS ligand and epithelial cell apoptosis. Furthermore, MMP7 is crucial in establishing chemokine gradients; *mmp7*^{-/-} mice are unable to effectively recruit neutrophils to sites of pathogen entry or tissue damage,⁴¹ suggesting an important role in inflammation. It is also responsible for activation of pro-TNF on macrophages⁴² and has been identified as a biomarker of idiopathic pulmonary fibrosis.⁴³

The role of MMPs in *Chlamydia*-induced diseases has been studied. MMP9 has received the most attention after reports that indicated increased conjunctival expression^{20,24} and enzymatic activity¹⁸ in trachoma patients. As yet, there are no small rodent models of ocular chlamydial infection that can reproduce the chronic changes observed in the human conjunctiva. Urogenital infection of the mouse offers the next best approximation in small rodents and does induce the expression of MMPs (MMP2, -9, and -12⁴⁴). Administration of chemical inhibitors of MMPs protected mice from ascending infection and hydrosalpinx.⁴⁵ Subsequent experiments in MMP9KO mice demonstrated that the ablation of MMP9 production was sufficient in this model to prevent the pathologic changes associated with ascending genital infection.⁴⁶ Changes in MMP7 levels have not been directly reported in response to chlamydial disease, although the effect of infection in MMP7KO mice has been studied in relation to sequelae and the immune response. Deficiency in MMP7 had little effect on the magnitude or duration of infection, fertility rates, and hydrosalpinx formation,⁴⁷ leading the authors to conclude that MMP7 was not a requirement in the progression or resolution of chlamyd-

ial infection in this model. However, the natural history of ocular infection in humans is markedly different from that in murine genital tract models of infection; thus, the contribution of MMP7 to fibrosis in trachoma is likely the result of repeated or prolonged inflammatory insults, which are often associated with colonization with multiple bacterial species.

MMP7 is one of several genes whose expression is controlled via Wnt (wingless, int) signaling. At least seven recognized terminal target genes of canonical Wnt signaling have been identified in fibrosis (*BMP*, *MYC*, *CD44*, *NOS2*, *MMP7*, *PPAR*, and *FNI*).⁴⁸ Four of these genes (*CD44*, *NOS2*, *MMP7*, and *FNI*) were upregulated in TT cases on the array. A limited number of genes involved in the Wnt signaling pathway were covered on the array, some of which are involved in E-cadherin signaling and the maintenance of the epithelial cell barrier.

Immune-mediated fibrotic diseases, such as schistosomula-induced hepatic fibrosis, are dominated by Th2-cytokine responses where IL13 and the decoy receptor IL13R α 2 are key.⁴⁹ IL-11 and its receptors are also clearly identified as playing a significant role in some immune-mediated fibrotic diseases.⁵⁰ More recently polarized macrophages that favor deposition of collagens and fibrosis have been demonstrated to be important in the development of disease (e.g., pulmonary fibrosis⁵¹ and responses to chronic helminth infection⁵²). There was no evidence of Th1/Th2/Th3 polarization, although altered expression of *NFATC1/4*, *SOC1*, and *TMED1* may suggest some evidence of inhibition of IFN γ signaling and stimulation of Th2, NK2 cells. We did not identify any of the factors found in previous studies on trichiasis patients as having significantly altered expression (*IL1B*, *TNF*, *MMP1*:*TIMP1*). Nor were genes

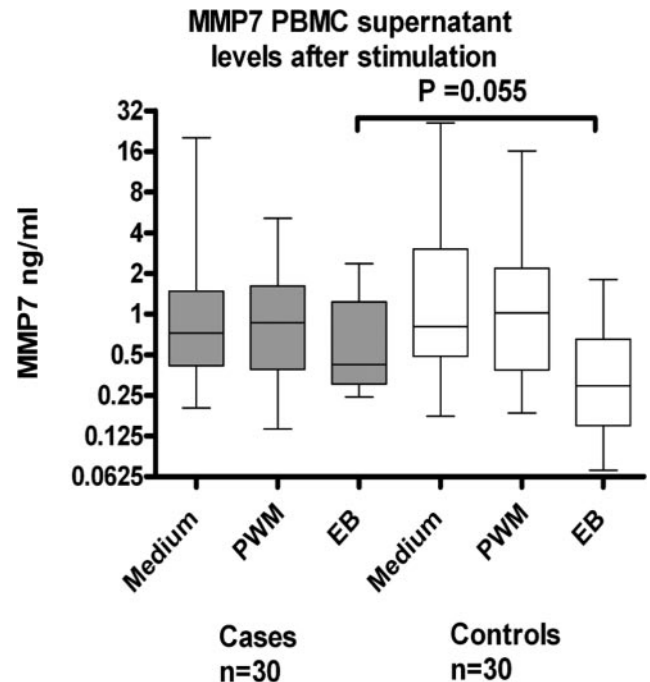


FIGURE 5. Production of MMP7 (ng/mL) in tissue culture supernatants collected from cases and controls. Freshly isolated PBMCs from cases or controls were cultured with PWM, serovar A EBs, or culture medium alone for 6 days. Production of MMP7 by cells in culture medium alone was the highest in both cases and controls. Stimulation with either PWM or EB inhibited the spontaneous production of MMP7 by PBMCs. This difference in levels reached borderline significance in response to stimulation with EBs, such that in cases (median [IQR] = 0.48 [1.52–0.35] ng/mL) stimulation with EBs has a lesser effect on the inhibition of MMP7 production than in controls (median [IQR] = 0.35 [0.62–0.128] ng/mL).

that have been identified in other infection or immune-driven fibrotic diseases differentially expressed. This does not rule out the involvement of these genes in the disease process. The small number of subjects studied and the sensitivity of the array could account for the lack of differences. In addition, the simplified WHO grading system used to identify cases and controls, although satisfactory for rapid population screening by national control programs, does not record additional important information for detailed research that may be required. Taken together, these factors may explain why these genes were not identified as differentially expressed. Within these limitations, the results identify the major changes in gene expression in this group of subjects and point to changes in MMP7 as the major gene with altered expression.

Since MMP7 gene expression was upregulated in the TT cases, we investigated whether the change would be reflected in the proteins recovered from the same conjunctival samples. The total protein showed some evidence of changes in the abundance of proteins below 60 kDa. This result warrants further study by two-dimensional SDS-PAGE. Using immunoblot analysis, we demonstrated the presence of MMP7, but attempts to quantify the amount of protein by capture ELISA were inhibited by the sample. The assay used to quantify MMP7 was developed to be performed in the presence of TIMP, the natural inhibitor of MMP7; thus, inhibition was due to an unknown factor. The lack of differences in MMP7 in pooled samples by immunoblot analysis may be masked the natural biological variation of the samples and the semiquantitative nature of immunoblot analysis techniques. Stimulation of PBMCs from cases and controls with *C. trachomatis* EBs resulted in a reduction in the inhibition of spontaneous production of MMP7 in the cases compared with that in the controls (i.e., higher levels of MMP7 remained in the supernatants of cases after stimulation). This observation is difficult to interpret and relate to the increased MMP7 gene expression observed in the conjunctiva of the TT cases. However, PBMCs are a recognized source of MMP7 in several studies, including the observation of differing levels of spontaneous production between clinical groups.^{53,54}

RT-PCR in 47 case-control pairs showed that *TLR6* expression was reduced in TT cases, suggesting that the host's recognition of bacterial ligands is affected in TT cases. Alternatively, reduced *TLR6* expression could be due to a reduced number of *TLR6*-expressing cells or changes caused by the disease process that result in the well-described increased susceptibility to other bacterial infections.⁵⁵ In the mouse, reduced *Tlr6* expression has been shown in macrophages on stimulation with TNF α or IFN γ ,⁵⁶ and a proinflammatory environment has also been shown to reduce *TLR6* expression in epithelial cells.⁵⁷

Reduced transcripts of two collagen proteins in subjects with scarring trachoma suggest that the composition of the extracellular matrix is altered. Collagen type I (*COL1A1*) and VII (*COL7A1*) transcripts were downregulated in individuals with TT. Many factors including cytokines affect expression of *COL1A1*. TGF β , IL-1 β , TNF α , and IFN γ have all been shown to affect expression of *COL1A1*.⁵⁸ *COL7A1* gene expression is also regulated by proinflammatory cytokines (TNF α , IL-1 β , and TGF β), expression is up- or downregulated depending on the cell-type and the cytokines present,^{59,60} indicating a complex biology. Transcription of *COL7A1* is important in the formation of anchoring fibrils, and this may play a role in the morphologic changes that take place in the conjunctiva of subjects with trichiasis.

C. trachomatis DNA was detected in only one individual, consistent with the low prevalence of *C. trachomatis* infection normally seen in subjects with TT in The Gambia and elsewhere.⁶¹ We did not collect additional swabs for standard

microbial cultures. Publications in the literature suggest that ~40% of subjects with TT are colonized with at least one nonchlamydial bacterial species.⁶² The contribution of additional species and changes in the ocular microbiome are therefore important, since they directly influence the host's expression profile and response to further challenge by infection.⁶³ The altered PBMC response levels of MMP7 in trichiasis may be a result of inherited genetic polymorphisms that predisposes the host to inflammation-driven fibrosis. Investigation of the signaling pathways and downstream targets of MMP7, along with MMP7 polymorphisms (some of which have already been identified as risk factors for fibrosis^{64,65}), may yield important information in the etiology of the scarring process.

We ultimately found four genes with altered expression: *MMP7*, *COL1A1*, *COL7A1*, and *TLR6*. We accepted a high rate of potential false-positive association when screening genes by the array and subsequently during validation by qRT-PCR, since the normal methods of correcting *P*-values and the general applicability of adjusting for multiplicity of tests remain matters of contention among biostatisticians.^{23,28} We therefore leave our colleagues to balance the significance of the findings against the interpretation of the study results. An independent replication study with a larger number of subjects studied by each method is the most appropriate manner of testing these associations. We suggest that in geographic regions where active trachoma and infection are hypoendemic, the *C. trachomatis*-specific adaptive and regulatory immune responses exert their major effect, if any, during the active stages of the disease when conjunctival scarring is in its early stages. The later stages of disease, such as those seen in adult TT cases, which are characterized by innate proinflammatory and fibrotic responses, continue to progress once the inflammatory environment is established and maintained by other nonchlamydial inflammatory insults. In populations, where rapidly advancing TS and TT can be observed in children and young adults, the role of *C. trachomatis*-infection and the inflammatory immune response in the acceleration of the disease process requires closer inspection with longitudinal follow-up of participants and repeated measures of gene expression.

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