Fibroblasts profiling in scarring trachoma identifies IL-6 as a functional component of a fibroblast-macrophage pro-fibrotic and pro-inflammatory feedback loop

Jenny Z. Kechagia1, Daniel G. Ezra1,2, Matthew J. Burton2,3 & Maryse Bailly1

Trachoma is a conjunctiva scarring disease, which is the leading infectious cause of blindness worldwide. Yet, the molecular mechanisms underlying progressive fibrosis in trachoma are unknown. To investigate the contribution of local resident fibroblasts to disease progression, we isolated conjunctival fibroblasts from patients with scarring trachoma and matching control individuals, and compared their gene expression profiles and functional properties in vitro. We show that scarring trachoma fibroblasts substantially differ from control counterparts, displaying pro-fibrotic and pro-inflammatory features matched by an altered gene expression profile. This pro-inflammatory signature was exemplified by increased IL-6 expression and secretion, and a stronger response to macrophage-mediated stimulation of contraction. We further demonstrate that scarring trachoma fibroblasts can promote Akt phosphorylation in macrophages in an IL-6-dependent manner. Overall this work has uncovered a distinctive molecular fingerprint for scarring trachoma fibroblasts, and identified IL-6- as a potential contributor to the chronic conjunctival fibrosis, mediating reciprocal pro-fibrotic/pro-inflammatory interactions between macrophages and fibroblasts.
conjunctiva outer layers and contain mixed cell populations, so may not accurately reflect changes occurring in the substantia propria – stroma – where the fibroblasts lie. Here we describe the phenotypic and genomic profile of fibroblasts isolated from conjunctival biopsies collected from Tanzanian individuals with scarring trachoma and from control subjects from the same area. We show that trachoma-derived fibroblasts display a classical pro-fibrotic phenotype, which is underlined by a specific gene expression profile. In addition, we identify IL-6 as a signature gene for scarring trachoma, and demonstrate that its main role is in modulating the local immune response, potentially contributing to the chronic activation of the fibroblasts.

Results
Trachoma-derived fibroblasts exhibit a higher ability to contract 3D collagen matrices. Primary fibroblast cell lines were established from trachomatous trichiasis (Scarring Trachoma Fibroblasts, STFs) and matching control conjunctival biopsies (Control Fibroblasts, CFs) with a similar success rate (12/26 and 10/20 for trachomatous trichiasis and control biopsies respectively, with similar age range and gender balance; SI Appendix, Table S1). There were no obvious morphological differences between the two sets (Fig. S1a) and they displayed comparable integrin expression profiles, suggesting a similar tissue origin (Fig. S1b). The primary fibroblasts cultures were expanded and frozen in aliquots to generate substantial stocks of cells at low passage. A subset of 8 control (C24, C26, C29, C30, C31, C103, C110, C111) and 8 trachoma (F07, F09, F10, F11, F12, F99, F106, F109) fibroblast cultures, for which we had the largest stocks, were chosen for further analysis. We used our standard in vitro collagen gel contraction assay to determine the contractile profile of STFs and CFs, in the presence of serum (10%) or cytokines and growth factors previously linked to active and scarring trachoma (PDGF-BB, TGF-β1, IL-1β, IL-17A, TNFα, CTGF, CXCL5, IL-1α, IL-1β, IL-17A, TNFα, CTGF, CXCL5)14. STFs were on average more contractile than CFs in all conditions, although this trend was only significant for serum and PDGF stimulation (Fig. 1a–e). Both STFs and CFs responded well to TGFβ1 and PDGF, but barely to IL-1b (Fig. 1c–e), and none of the other cytokines tested had any effect on contraction (not shown). There was no difference in cell viability/proliferation between CFs and STFs during contraction in the presence of serum (Fig. 1f), suggesting that the increased contraction ability displayed by STFs is an intrinsic cell feature and not a consequence of altered proliferation and/or survival.

Trachoma-derived fibroblasts display a mildly altered matrix remodelling phenotype. Fibrotic tissue is characterized by increased matrix remodelling including both neo-matrix synthesis and matrix degradation, and we have shown that matrix remodelling is an essential component of fibroblast-mediated gel contraction11,12. We thus sought to determine whether exaggerated matrix remodelling properties could underlie STF contractile phenotype. Control and trachoma fibroblasts displayed similar morphological features in collagen gels during contraction (Fig. S2). Confocal reflection microscopy revealed a small, but consistent, increase in contractile phenotype. Control and trachoma fibroblasts displayed similar morphological features in collagen gels during contraction (Fig. S2). Confocal reflection microscopy revealed a small, but consistent, increase in contractile phenotype. Control and trachoma fibroblasts displayed similar morphological features in collagen gels during contraction (Fig. S2). Confocal reflection microscopy revealed a small, but consistent, increase in contractile phenotype. Control and trachoma fibroblasts displayed similar morphological features in collagen gels during contraction (Fig. S2).

Trachoma-derived fibroblasts display altered contraction force and tissue mechanics. Alpha smooth-muscle actin (α-SMA) expression is one of the most well characterized markers of fibroblast activation towards the contractile myofibroblast phenotype commonly encountered in fibrosis. Using immunofluorescence to analyse α-SMA expression during contraction, we could not detect any α-SMA expression at day 1 of culture. The proportion of α-SMA positive fibroblasts increased thereafter to about 40–50% α-SMA positive cells at day 7. Again, although STFs had on average slightly more α-SMA positive cells, the difference was not significant (Fig. S3). We have shown previously a link between fibroblasts’ contraction potential and intrinsic cellular force levels9,13. To determine whether STFs displayed altered cellular force, we used the PalpatorTM (InvivoSciences, USA), an automated force measurement platform, to measure cellular contractile force and matrix stiffness in pre-stressed 3D collagen hydrogels populated with STFs and CFs. The cells were embedded in collagen gels in customized supports allowing gel contraction under tensioned conditions, leading to the formation of a tissue-like structure where the cells establish tensional homeostasis14. There was no difference in the ability of STFs and CFs to generate tissue constructs, and they displayed similar levels of cell force at resting state (after 48 hours starvation in serum-free medium, Fig. 2c,d). Likewise, both cell types displayed a significant and comparable increase in cell force (by about 40%, p < 0.01) upon stimulation with 20% serum. However, while the force remained stable for CFs for up to 45 min (Fig. 2c), it was only transient in STFs, suggesting altered cellular force responses (Fig. 2d).

To further explore the mechanical properties of the tissue constructs, we used the matrix force properties acquired with the Palpator to extract the elastic modulus (E), after calculating the cross sectional area of the collagen constructs14. We found that tissue constructs generated by CFs and STFs had levels of stiffness between 1 and 4 kPa, within the range of that of intact connective tissue15. However, tissues generated by STFs were significantly stiffer, with an Elastic Modulus value of about twice that of the tissues generated by CFs (Fig. 2c).

Trachoma-derived fibroblasts display a specific pro-inflammatory and pro-fibrotic gene expression profile. To identify molecular markers associated with the pro-fibrotic profile of STFs, we performed a comparative gene expression analysis of STFs and CFs using a microarray platform (Affymetrix human gene 1.0 ST). Data analysis was done using AltAnalyze (http://www.altanalyze.org/), and the raw Affymetrix CEL files were processed using the Robust Multi-array Average (RMA) normalization methodology16. Due to the small sample size, we conducted a moderate t-test, as calculated by limma’s R package. We found that 128 genes were upregulated and 46 were downregulated in STFs compared to CFs, exhibiting more than 2 fold change (FC) and P

Data analysis was done using AltAnalyze (http://www.altanalyze.org/), and the raw Affymetrix CEL files were processed using the Robust Multi-array Average (RMA) normalization methodology16. Due to the small sample size, we conducted a moderate t-test, as calculated by limma’s R package. We found that 128 genes were upregulated and 46 were downregulated in STFs compared to CFs, exhibiting more than 2 fold change (FC) and P
values <0.05 (Table S3). Figure 3A illustrates the hierarchical clustering of gene expression patterns shared by the samples. The principal component analysis confirmed tight clustering of the control samples (Fig. 3b). There was more variability within the trachoma samples, with F11 in particular clustering closer to controls.

GO-Elite software was used to identify a non-redundant set of ontology terms and pathways enriched in STFs. Gene ontology revealed genes associated with extracellular matrix organization, positive regulation of inflammatory response, developmental process, and Wnt signalling. Overall, the molecular processes enriched in our dataset can be divided into three different categories: pro-inflammatory, pro-fibrotic and genes associated with cell differentiation and development (Fig. 3c,d, Table S4). In addition, pathway analysis highlighted the involvement of cell differentiation processes and enrichment of genes related with TGF-b pathway, drawing a pro-fibrotic gene expression profile (Table S6). Using RT-qPCR analysis, we validated the differential expression of 6 of the most affected genes (NCAM2, TFAP2A, IL-6, PCDH7, THBS4 and WISP3), confirming a significantly altered genomic expression profile in STFs (Fig. 4a).

**IL-6 production by trachoma-derived fibroblasts contributes to macrophage activation.** Amongst the genes identified as differentially expressed in STFs and implicated in most pathways, particularly

**Figure 1. Scarring trachoma fibroblasts display increased matrix contraction abilities.** Control (CFs) and trachoma-derived (STFs) fibroblasts were embedded in collagen matrix and contraction was measured over a 7-day period, in medium with 10% serum (FBS), serum-free medium (SF), or SF with cytokines (PDGF-BB 10 μg/mL, TGF-b 5 μg/mL and IL-1b 10 ng/mL). (a–e) STFs exhibited higher contraction compared to CFs in the presence of serum (**p = 0.0025, two-tailed t-test), or PDGF-BB (*p = 0.0438). Shown is mean ± SEM for gel contraction at day 7 (each data point as the average of 3–4 experiments with triplicate gels for 8 STFs and 8 CFs). (f) CFs and STFs have similar metabolic activity at days 1 and 4 of contraction, as measured with an Alamar blue fluorescence assay (mean ± SEM for n = 3 for 4 CFs and 4 STFs).
noticeable was the prominent place of IL6 (Fig. 3, Tables S3 and S4). IL-6 is an important mediator of inflammation and a previously suggested risk factor for scarring trachoma. IL6 expression was found to be increased 6-fold in STFs compared to CFs in the microarray study, and this increase was validated using real-time qPCR (Fig. 4a). An ELISA analysis of the medium during contraction in the presence of 10% serum confirmed significantly higher IL-6 levels for STFs (Fig. 4b). To investigate whether this increased IL-6 expression could be linked to STFs’ contractile potential, we used blocking antibodies and siRNA to neutralise or prevent IL-6 release during contraction. Despite significantly blocking IL-6 function and expression respectively (Fig. S4a), neither antibody nor siRNA treatment affected CFs or STFs’ contraction (Fig. 4c). Likewise, IL-6 did not substantially stimulate contraction (Fig. 4d).

Figure 2. Scarring trachoma fibroblasts display altered force responses and matrix remodelling properties. (a,b) CFs and STFs (8 different cell lines each) were embedded in collagen gels for contraction in the presence of 10% serum and the culture medium was collected on days 3 and 5 for measurement of (a) MMP release (Active fraction and total amount following APMA activation), and (b) day 3 to day 7 for neo-collagen synthesis. Shown are the means +/- SEM of 8 CFs and 8 STFs (n = 2–3 for each cell line). (c–e) CFs (c) and STFs (d) were embedded in collagen gels in MC-8™ micro-chambers and cultured for 2 days to generate tissues. The tissues were starved for 48 hours and cellular force was measured every 15 min after the addition of 20% serum. A mock stimulated control set (Starved) was done in parallel for each experiment. Shown is cell force, with mean +/- SEM for n = 1–3 for 8 CFs and 8 STFs (***p < 0.01, *p < 0.05, significantly different from time 0, 2-way ANOVA, Benferroni post-test). (e) Force measurements were acquired for CFs (C24, C30, C31) and STFs (F10, F11, F12) as in (c,d) above and the Elastic modulus (E) of the tissues was extracted from the matrix force formula F_m = E x A, where A is the tissue cross-section area. Shown is the mean +/- SEM (n = 4 for each cell line with 3 time points upon stimulation; *p = 0.0152, two-tailed t-test).
Scarring in trachoma is often characterised by an increased inflammatory infiltrate, including macrophages and lymphocytes. Owing to the strong involvement of IL-6 in the pro-inflammatory expression profile of STFs, we hypothesized that the increase in IL-6 expression in STFs might be linked to inflammation. We produced differentiated macrophages from the U937 monocyte human cell line using PMA stimulation, and co-cultured them with CFs or STFs within collagen gels. Macrophages alone did not induce any contraction (Fig. S4b), and in the presence of 10% serum did not affect fibroblast-mediated gel contraction, even at a fibroblast:macrophage ratio of 1:4 (Fig. 5a). However, in the absence of serum, macrophages strongly stimulated fibroblast-mediated collagen contraction (Fig. 5b). As for serum conditions, macrophages alone did not cause any contraction (Fig. S4b). Importantly, the increase in contraction upon co-culture with macrophages was higher for STFs compared to CFs (p < 0.05). To test whether this difference could be linked to the increased IL-6 production in STFs compared to CFs (p < 0.05). To test whether this difference could be linked to the increased IL-6 production in STFs compared to CFs. To test whether this difference could be linked to the increased IL-6 production in STFs compared to CFs.

Discussion
Trachoma is a major cause of blindness worldwide. Despite being one of the oldest diseases ever recorded, our knowledge of the mechanism of scar tissue formation, responsible for the devastating blindness consequences, is still limited. While this may be partly attributed to the lack of a suitable animal model, the research so far has focused on the complex immune responses, with little attention being paid to the local tissue stroma. Previous studies, using conjunctival swabs from patients with trichiasis, have identified a number of genes potentially underlying the fibrotic phenotype in trachoma. These included keratinization markers and classical inflammation...
components, such as *IL1B*, *CXCL5*, *INDO* and *S100A7*, as well as MMPs. However, such samples are often inflamed and contain a large fraction of the epithelium and immune cells, making it difficult to identify the contribution of the underlying fibroblasts. Previous work from our group and others have shown that fibroblasts isolated from ocular fibrotic tissue retain pro-fibrotic features and distinct genomic profiles in vitro, allowing the identification of putative novel disease pathways. Here we used a similar in vitro system to characterise some of the key features of scarring trachoma fibroblasts and investigate their contribution to disease progression.

We found that trachoma fibroblasts were more efficient at contracting collagen gels, and displayed altered cell force responses and increased matrix remodelling properties compared to their normal counterparts. Moreover, they were both more responsive to macrophage-derived stimuli and more stimulatory towards macrophages. This pro-fibrotic and pro-inflammatory phenotype was maintained in vitro for several passages, indicating a genuine alteration of the resident tissue fibroblasts in trachoma. In agreement with that, we identified a unique pro-inflammatory and pro-fibrotic gene expression signature in scarring trachoma fibroblasts, suggesting that the observed phenotypic differences are underpinned by true differences in gene expression.

**Pro-fibrotic profile of scarring trachoma fibroblasts.** Tissue homeostasis is perturbed in fibrotic conditions, where altered matrix expression and remodelling, accompanied by an increased contractility in resident fibroblasts often lead to changes in tissue mechanics and stiffness. This increase in contracture has most often been attributed to myofibroblast differentiation, as defined by α-SMA expression. However, in agreement with our previous work on ocular fibroblasts and other studies, we found that trachoma-derived fibroblasts did not display a significant increase in α-SMA gene expression, nor do they express the protein in high amounts during contraction in vitro. Accordingly, they did not show higher contractile force, whether at resting state or in response to serum, which classically stimulates acto-myosin contraction. Their force response was nevertheless clearly different in terms of dynamics, possibly indicating an adaptation to growth factor stimulation as a result of increased matrix stiffness and changes in matrix composition, as we previously reported for other fibroblasts.

**Figure 4.** IL-6 is overexpressed in scarring trachoma fibroblasts but does not directly affect matrix contraction. (a) Sample genes from the microarray were validated using quantitative real-time PCR using RNA extracted from 6 CFs (C03, C24, C29, C30, C103, C111) and 7 STFs (F07, F09, F109, F99, F11, F106, F109) (n = 2–3 repeats each). Results are expressed as fold change over CFs samples, following normalization to GAPDH expression (mean +/- SEM). (b) CFs and STFs were embedded in collagen gels and allowed to contract in the presence of 10% serum. IL-6 levels were measured at day 7 using ELISA. Graph shows the mean +/- SEM for 7 CFs and 8 STFs cell lines (n = 2 for each; *p = 0.0249; two-tailed t-test). (c) Inhibition of IL-6 using neutralizing antibody (IL-6 nAb; 300 ng/ml) or siRNA (IL-6 siRNA) did not alter CFs or STFs contraction compared to control immunoglobulin (IgG) or Non-target control siRNA (NT-siRNA) respectively. Graph shows the mean +/- SEM for contraction at day 7 in presence of 10% serum (n = 2–3 for 4 CFs and 3 STFs). (d) IL-6 (20 ng/ml) did not significantly stimulate contraction. Graph shows mean +/- SEM for gel contraction at day 7, in serum free (SF) medium (n = 2 for 4 CFs and 4 STFs).
of the long-term chronic stimulation in vivo. This is consistent with the expression analysis, which identified various component of the cytoskeleton (both actin and microtubules) and related signalling pathways (including small Rho GTPases regulators) altered in trachoma cells, suggesting that their altered force profile may be due to modified cytoskeletal dynamics downstream receptor activation. GO analysis indicated associations with genes related to muscle (e.g calcitonin-like receptor; dystrophin, Plakophilin), contraction, actin cytoskeleton, cell-cell interactions and cell migration in STFs, possibly supporting the pro-contractile phenotype. Interestingly, it also
suggested an apparent differential regulation of microtubules, which has recently been suggested as a way by which Chlamydia infection could reprogram the host cell cytoskeleton\(^{34}\). In addition, GO analysis of our dataset partially overlaps with a recent genome-wide association study (GWAS) of scarring trachoma\(^{36}\). Thus the trachoma fibroblast signature we identified may reflect a combination of scar-driving alterations following early infection, as well as consequences of the chronic scarring.

When prompted to generate tissue-like structures through contraction of tethered gels, both control fibroblasts and STFs established tensional homeostasis at stiffness levels within the range of that expected of a typical stromal tissue\(^{15,27}\). However, the elastic modulus of the tissues generated by STFs was significantly higher, potentially mirroring the fibrotic origin of the cells. This may reflect an adaptation to the fibrotic environment\(^{38}\), and/or a change in mechanostat level\(^{39}\). Noticeably, the stiffness levels reached by STF-derived tissues were still an order of magnitude lower (in the kPa range) than the Elastic modulus values reported for fibrotic tissue, usually in the 10kPa range and over\(^{29}\). This suggests that although alterations to the fibroblasts mechanical properties may contribute to the development of the fibrotic tissue in trachoma, other components, e.g. epithelial layer and inflammation, are needed to recreate the full pathology and subsequent tissue alterations. It is nevertheless possible that because of their altered mechanical properties, the trachoma-derived fibroblasts directly promote fibrosis in vivo by sustaining a stiffness-increasing loop, as proposed for lung fibrosis\(^{30}\) or cancer\(^{31}\). This is supported by the expression array analysis, which revealed alterations in several molecules involved in matrix synthesis and crosslinking in scarring fibroblasts. These include Pro-collagen-l, 2-oxoglutarate 5-dioxygenase 2 (PLOD2) and collagen modifying molecule leprecan-like 1 (LEPREL1 or LEPREL1P3H2), two enzymes involved in post-translational collagen modification and crosslinking\(^{22,33}\). Both are significantly upregulated in scarring trachoma fibroblasts (Table S3), and could contribute to matrix stiffening. The array also suggested an upregulation of matrix synthesis functions in scarring fibroblasts, exemplified by an upregulation of collagen type IV (Table S3), as previously observed in vivo\(^{34}\).

Pro-Inflammatory profile of scarring trachoma fibroblasts. Scarring trachoma is often accompanied by a sustained inflammatory infiltrate marked by a significant contribution of the innate immune responses, including macrophages. The presence of inflammation is linked to a high recurrence rate following trichiasis surgery\(^{3,35}\), suggesting that macrophages may contribute to fibroblasts’ stimulation towards scarring. Macrophages did not noticeably affect control or scarring trachoma fibroblast-mediated contraction in the presence of serum, suggesting that the fibroblast’s contractile behaviour is dominated by the response to the biomechanical environment and the mixed growth factor/cytokine stimulation provided by the serum. However, in the absence of serum, macrophages fully stimulated contraction, albeit to a higher degree in scarring trachoma cells, suggesting that diseased fibroblasts interact differently with macrophages. Indeed, our array analysis identified an overall pro-inflammatory profile in STFs, including genes previously implicated in fibrotic conditions and autoimmune disorders such as toll-like receptor 4 (TLR4), heme oxygenase 1 (HMOX1) and the TNF ligand superfamily member 4 (TNFSF4)\(^{36,37}\), and we were able to show that IL-6 secreted by STFs specifically altered macrophage responses. IL-6 is a pleiotropic molecule with diverse roles in health and disease and a driving component in fibrosis\(^{38,39}\). IL-6 levels are elevated in the conjunctiva mucosal secretions of patients with trachomatous trichiasis, leading to the suggestion that IL-6 may be a prognostic marker for scarring trachoma, but the exact cellular origin of IL-6 in such secretions is unclear\(^{40}\). We showed here that IL-6 expression and secretion were up-regulated in STFs, but did not play a direct role in the contraction phenotype, even in the presence of macrophages. Rather, it contributed to an increase in Akt phosphorylation in macrophages, which has been linked to cell survival\(^{30}\). Thus, increased IL-6 secretion in trachoma may sustain local macrophage survival and activation, which in turn could further the contraction activity of the fibroblasts. We propose that such a “fibrosis activation feed-back loop” could sustain recurrent scarring in trachoma.

Cell differentiation and neuronal profile. In addition to pro-fibrotic and pro-inflammatory features, the profiling of scarring trachoma fibroblasts revealed a strong involvement of genes linked to differentiation and developmental processes, including Wnt and TGF-β signalling pathways, confirming previous studies\(^{8,26}\). These pathways are essential during embryogenesis and development, and their interaction and activation in differentiated cells can result in permanent changes in gene expression leading to disease, including fibrosis\(^{40–43}\). Amongst the genes implicated was WNT2B, which has been shown to trigger canonical Wnt signalling responses\(^{41}\), as well regulate fibrotic and developmental responses\(^{41,42}\). Its up-regulation in our dataset (Table S3) might indicate a possible role of canonical Wnt signalling in scarring trachoma. Neuronal differentiation markers were also prominent in our GO analysis as well as in the GWAS study\(^{36}\), including Neural Cell Adhesion Molecule 2 (NCAM2) and transcription factor activating protein 2 a (TFAP2A). NCAM2 is predominantly expressed in the brain and stimulates neurite outgrowth and axonal compartmentalization\(^{44}\), while TFAP2A has been implicated in eye development\(^{45}\) and neuronal stem cell differentiation of mesenchymal stem cells\(^{46}\). This suggests a predisposition for transdifferentiation towards neuronal lineage in scarring trachoma, which may partly be down to the neuronal crest origin of the facial fibroblasts\(^{47}\).

Conclusion Overall we have identified clear phenotypic and gene expression alterations in scarring trachoma fibroblasts, which may underlie the disease pathology. While overlapping with classical pro-fibrotic and pro-inflammatory pathways, the profile of trachoma fibroblasts bears unique features, possibly linked to early events in the disease progression. Partial overlapping of gene ontology with a published GWAS study\(^{36}\) suggests that there might be some contribution of genetic factors to the scarring fibroblasts’ phenotype. Alternatively, some of the expression changes could be due to epigenetic modifications, e.g methylation, following early infection events and/or chronic inflammation. Similarly, the cells’ matrix contraction ability was not directly linked to classical contractile pathways, the profile of trachoma fibroblasts bears unique features, possibly linked to early events in the disease progression. Partial overlapping of gene ontology with a published GWAS study\(^{36}\) suggests that there might be some contribution of genetic factors to the scarring fibroblasts’ phenotype. Alternatively, some of the expression changes could be due to epigenetic modifications, e.g methylation, following early infection events and/or chronic inflammation. Similarly, the cells’ matrix contraction ability was not directly linked to classical contractile pathways, the profile of trachoma fibroblasts bears unique features, possibly linked to early events in the disease progression. Partial overlapping of gene ontology with a published GWAS study\(^{36}\) suggests that there might be some contribution of genetic factors to the scarring fibroblasts’ phenotype. Alternatively, some of the expression changes could be due to epigenetic modifications, e.g methylation, following early infection events and/or chronic inflammation. Similarly, the cells’ matrix contraction ability was not directly linked to classical contractile
markers (e.g. α-SMA expression) or inflammatory stimulus (e.g. TGFβ). Rather, more subtle changes in cell dynamics are likely to underlie the contraction phenotype, and classic pro-inflammatory cytokines may promote the scarring indirectly, through reciprocal interactions with inflammatory cells. Indeed, we have identified an IL-6-driven pro-fibrotic/pro-inflammatory feedback loop that may contribute to chronic scarring in trachoma, possibly underlying the recently uncovered link between clinical inflammation and progressive scarring. This suggests that conjunctival stroma may play a more central role than previously thought in the development of the disease and opens new avenues in the design of novel therapeutic targets in scarring trachoma.

Materials and Methods

Detailed Material and Methods and reagents are provided in the SI Appendix, Materials and Methods.

Cells. Primary fibroblasts were expanded from biopsies obtained from the upper tarsal conjunctiva of Tanzanian patients undergoing trichiasis surgery and control individuals undergoing cataract surgery. This study adhered to the tenets of the Declaration of Helsinki, and was approved by the Tanzanian National Institute of Medical Research, the Kilimanjaro Christian Medical Centre, and the London School of Hygiene and Tropical Medicine Ethics Committees. The study was explained to potential study participants and written informed consent was obtained before enrolment. The cells were maintained in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% (v/v) heat-inactivated foetal bovine serum, 100 IU/ml penicillin, 100 μg/ml streptomycin and 25 μg/ml gentamycin. Collagen matrices were supplemented with 2% (v/v) heat-inactivated foetal bovine serum, 100 IU/ml penicillin, 100 μg/ml streptomycin and 25 μg/ml gentamycin. Cells were maintained in culture until 70% confluence and were then used in experiments (Supplementary Figure S1).

Collagen contraction. Fibroblast-mediated matrix contraction was assessed using free-floating collagen gel lattices, as described before. To determine cell response to cytokines and growth factors, gels were made using serum free (SF) medium with the addition of one of the following factors: IL-1β, TGF-β1, TNF-α, CTGF or CXCL5 (5 ng/ml, Peprotech, UK), IL-6 (20 ng/ml, R&D Systems, UK). For co-culture experiments, macrophages were added to the gel mix at a final ratio of 1 (fibroblast):4 (macrophages). Cell metabolic activity in gels was measured using 10% Alamar blue. MMP activity was measured at day 5 using an MMP activity assay kit according to manufacturer guidelines (Abscam, UK). Supernatants were either incubated for 2 hours with 2 mM APMA (4-aminophenylmercuric acetate) to allow activation of pro-MMPs for total MMP activity or used immediately to measure only active MMPs. An enzyme immunoassay (TaKara BIO INC.) was used to determine Pro-collagen Type I C-peptide release.

Force Measurements. Cell and Matrix force were calculated using the Palpatorm Force measurement platform (InvivoSciences LLC, McFarland, WI). Gels were made as described elsewhere using 4 × 10⁵ cells/ml. The collagen-cell mixture was poured into each well of an eight-well polycarbonate mould (8-well MC-8⁰⁰ chamber). The matrices were then cultured in complete DMEM at 37 °C for 2 days and starved in serum free medium for one day. Force measurements were taken every 15 min before and after stimulation with 20% FBS for a total of 45 min. A custom Matlab algorithm was used to process and analyse the force data to report a numerical parameter that is indicative of the active cell force in the tissue.

RNA Isolation and Affymetrix GeneChip Processing. RNA extraction was performed on 4 trachoma-derived (F07, F10, F11, F12) and 4 control (C24, C26, C29, C30) fibroblast cell lines (SI Appendix, Table S1) in standard cultures at passages 3 to 6, using an RNaseasy kit (Qiagen, UK), with 2 from each group done in independent duplicates (F07, F10 and C26, C30). The resulting RNA samples were then processed and analysed on a cDNA microarray platform (GeneChip Human Gene 1.0ST; Affymetrix, Santa Clara, CA) at the UCL Genomics microarray laboratory, Institute of Child Health (London, UK).

Microarray Analysis and validation. Gene array .cel files were processed using Genespring (GX version 10.01; Agilent), and data analysis was performed using AltAnalyze. Summarization and normalization of the gene array cel files was performed using the Robust MultiArray Average (RMA) method. Gene expression levels (Ensembl genes) and GO annotation with GO-Elite software were carried out, using default options. Genes differentially expressed were identified using a combination of a >2-fold change in expression and a significance of P < 0.05 (Lima’s, Student’s t-test). Validation was performed using qRT-PCR on a real-time PCR system (HT7900 Fast Real-Time PCR; Applied Biosystems) using GAPDH as a reference endogenous control. Western blot validation was performed as previously described using the following antibodies: IL-6, Mouse IgG1 (R&D systems); Actin, p-Akt (Ser473) (D9E) XP, XP, Stat3 (124H6), p-Stat3 (Tyr705) (D3A7) XP® (Cell Signalling). IL-6 secretion was validated using the Human IL-6 Quantitative ELISA Kit (R&D Systems) according to the manufacturers protocol.

References
1. Alliance for the Global Elimination of Blinding Trachoma by the year 2020. World Heal. Organ. Wkly. Epidemiol. Rec. 89(39), 421–428 (2014), at <http://www.who.int/whr/2013/whr8939.pdf>.
2. Hu, V. H., Hollander, M. J. & Burton, M. J. Trachoma: protective and pathogenic ocular immune responses to Chlamydia trachomatis. PLoS Negl. Trop. Dis. 7, e2020 (2013).
3. Burton, M. J. et al. Pathogenesis of progressive scarring trachoma in Ethiopia and Tanzania and its implications for disease control: two cohort studies. PLoS Negl. Trop. Dis. 9, e0003763 (2015).
4. Natividad, A. et al. Human conjunctival transcriptome analysis reveals the prominence of innate defense in Chlamydia trachomatis infection. Infect. Immun. 78, 4895–911 (2010).
5. Burton, M. J. et al. Active trachoma is associated with increased conjunctival expression of IL17A and profibrotic cytokines. Infect. Immun. 79, 4977–83 (2011).
6. Taylor, H. R. Ocular Models of Chlamydial Infection. Rev. Infect. Dis. Vol. 7, No. 6 737–740 (1985). at <http://www.ncbi.nlm.nih.gov/pmc/articles/PMC1617736/>

7. Abu el-Asrar, A. M. et al. Immunopathogenesis of conjunctival scarring in trachoma. Eye (Lond). 12 (Pt 3a), 453–60 (1998).

8. Burton, M. J. et al. Conjunctival transcriptome in scarring trachoma. Infect. Immun. 79, 499–511 (2011).

9. Erza, D. G., Ellis, J. S., Beaconsfield, M., Collin, R. & Bailly, M. Changes in fibroblast mechanostat set point and mechanosensitivity: an adaptive response to mechanical stress in floppy eyelid syndrome. Invest. Ophthalmol. Vis. Sci. 51, 3853–63 (2010).

10. Li, H., Erza, D. G., Burton, M. J. & Bailly, M. Doxycycline prevents matrix remodeling and contraction by trichiasis-derived conjunctival fibroblasts. Invest. Ophthalmol. Vis. Sci. 54, 4675–82 (2013).

11. Tovell, V. E., Chau, C. Y., Khaw, P. T. & Bailly, M. Rac1 inhibition prevents tissue contraction and MMP mediated matrix remodeling in the conjunctiva. Invest. Ophthalmol. Vis. Sci. 53, 6682–91 (2012).

12. Martin-Martin, B., Tovell, V., Dahlmann-Noor, A. H., Khaw, P. T. & Bailly, M. The effect of MMP inhibitor GM6001 on early fibroblast-mediated collagen matrix contraction is correlated to a decrease in cell protrusive activity. Eur. J. Cell Biol. 90, 26–36 (2011).

13. Dahlmann-Noor, A. H., Martin-Martin, B., Eastwood, M., Khaw, P. T. & Bailly, M. Dynamic protrusive cell behaviour generates force and drives early matrix contraction by fibroblasts. Exp. Cell Res. 313, 4158–69 (2007).

14. Marquez, J. P. et al. High-throughput measurements of hydrogel tissue construct mechanics. Tissue Eng. Part C. Methods 15, 181–90 (2009).

15. Butcher, D. T., Alliston, T. & Weaver, V. M. A tense situation: forcing tumour progression. Nat. Rev. Cancer 9, 108–22 (2009).

16. Irizarry, R. A. et al. Exploration, normalization, and summaries of high density oligonucleotide array probe level data. Biostatistics 4, 249–64 (2003).

17. Zambron, A. C. et al. GO-Elite: a flexible solution for pathway and ontology over-representation. Bioinformatics 28, 2209–10 (2012).

18. Skworetz, T. et al. Role of secreted conjunctival mucosal cytokine and chemokine proteins in different stages of trachomatous disease. PLoS Negl. Trop. Dis. 2, e264 (2008).

19. Hu, V. H. et al. In vivo confocal microscopy and histopathology of the conjunctiva in trachomatous scarring and normal tissue: a systematic comparison. Br. J. Ophthalmol. 97, 1333–7 (2013).

20. Chen, R. H., Chang, M. C., Su, Y. H., Tsai, Y. T. & Kuo, M. L. Interleukin-6 inhibits transforming growth factor-beta-induced apoptosis through the phosphatidylinositol 3-kinase/Akt and signal transducers and activators of transcription 3 pathways. J. Biol. Chem. 274, 23013–9 (1999).

21. Saw, V. P. J. et al. Profibrotic phenotype of conjunctival fibroblasts from mucous membrane pemphigoid. Am. J. Pathol. 178, 187–97 (2011).

22. Klingberg, E., Hinz, B. & White, E. S. The myofibroblast matrix: implications for tissue repair and fibrosis. J. Pathol. 229, 298–309 (2013).

23. Gabbiani, G. The role of contractile proteins in wound healing and fibrocontractive diseases. Methods A 97, 187–206 (1999).

24. Tomasek, J. J., Gabbiani, G., Hinz, B., Chapronnier, C. & Brown, R. A. Myofibroblasts and mechano-regulation of connective tissue remodelling. Nat. Rev. Mol. Cell Biol. 3, 349–63 (2002).

25. Dumoux, M., Menny, A., Delacour, D. & Hayward, R. D. A Chlamydia effector recruits CEPI70 to reprogram host microtubule organization. J. Cell Sci. 128, 3420–3434 (2015).

26. Roberts, C. H. et al. Conjunctival fibrosis and the innate barriers to Chlamydia trachomatis intracellular infection: a genome wide association study. Sci. Rep. 5, 17447 (2015).

27. Paszek, M. J. et al. Tensional homeostasis and the malignant phenotype. Cancer Cell 8, 241–54 (2005).

28. Brown, R. A., Prajapati, R., McGrother, D. A., Yannas, I. V. & Eastwood, M. Tensional homeostasis in dermal fibroblasts: mechanical responses to mechanical loading in three-dimensional substrates. J. Cell. Physiol. 175, 323–32 (1998).

29. Wells, R. G. Tissue mechanics and fibrosis. Biochem. Biophys. Acta 1832, 884–90 (2013).

30. Liu, F. et al. Feedback amplification of fibrosis through matrix stiffening and COX-2 suppression. J. Cell Biol. 190, 693–706 (2010).

31. Calvo, F. et al. Mechanotransduction and YAP-dependent matrix remodelling is required for the generation and maintenance of cancer-associated fibroblasts. Nat. Cell Biol. 15, 637–46 (2013).

32. van der Slot, A. J. et al. Identification of PLOD2 as telopeptide lysyl hydroxylase, an important enzyme in fibrosis. J. Biol. Chem. 287, 40967–72 (2003).

33. Fernandes, R. J., Farnaud, A. W., Traeger, G. R., Weiss, M. A. & Eyre, D. R. A role for prolyl 3-hydroxylase 2 in post-translational modification of fibril-forming collagens. J. Biol. Chem. 286, 30662–9 (2011).

34. Abu el-Asrar, A. M. et al. An immunohistochemical study of collagens in trachoma and vernal keratoconjunctivitis. Eye (Lond). 12 (Pt 6), 1001–6 (1998).

35. Burton, M. J. et al. Conjunctival expression of matrix metalloproteinase and proinflammatory cytokine genes after trichiasis surgery. Invest. Ophthalmol. Vis. Sci. 51, 3583–90 (2010).

36. Lei, W. et al. The upregulated expression of OX40/OX40L and their promotion of T cells proliferation in the murine model of asthma. J. Thorac. Dis. 6, 979–87 (2014).

37. Gourh, P. et al. Association of TNFSF4 (OX40L) polymorphisms with susceptibility to systemic sclerosis. Ann. Rheum. Dis. 69, 550–5 (2010).

38. Fielding, C. A. et al. Interleukin-6 signaling drives fibrosis in unresolved inflammation. Immunology 40, 40–50 (2014).

39. Le, T.-T. T. et al. Blockade of IL-6 Trans Signaling Attenuates Pulmonary Fibrosis. J. Immunol. 193, 3755–3768 (2014).

40. Guo, X. & Wang, X.-F. Signaling cross-talk between TGF-beta/BMP and other pathways. Cell Res. 19, 71–88 (2009).

41. Katoh, M., Kirikoshi, H., Terasaki, H. & Shiokawa, K. WNT2B2 mRNA, up-regulated in primary gastric cancer, is a positive regulator of the WNT-beta-catenin-TCF signaling pathway. Biochem. Biophys. Res. Commun. 289, 1093–8 (2001).

42. Akhmetshina, A. et al. Activation of canonical Wnt signalling is required for TGF-β-mediated fibrosis. Nat. Commun. 3, 7735 (2012).

43. Sun, Z. et al. Activated Wnt signalling induces myofibroblast differentiation of mesenchymal stem cells, contributing to pulmonary fibrosis. Int. J. Mol. Med. 33, 1097–109 (2014).

44. Winther, M., Berezin, V. & Walmrod, P. S. NCAM2/OCAM/RNCA1: cell adhesion molecule with a role in neuronal compartmentalization. Int. J. Biochem. Cell Biol. 44, 441–6 (2012).

45. Nottoli, T., Hagopian-Donaldson, S., Zhang, J., Perkins, A. & Williams, T. AP-2 null cells disrupt morphogenesis of the eye, face, and limbs in chimeric mice. Proc. Natl. Acad. Sci. 95, 13714–13719 (1998).

46. Bi, Y. et al. AP2α transcriptional activity is essential for retinoid-induced neuronal differentiation of mesenchymal stem cells. Int. J. Biochem. Cell Biol. 46, 148–60 (2014).

47. Creuzet, S., Vincent, C. & Couly, G. Neural crest derivatives in ocular and periorcular structures. Int. J. Dev. Biol. 49, 161–71 (2005).

Acknowledgements

This work was supported by Fight for Sight (Studentship 1897), the Special Trustees of Moorfields Eye Hospital (Ref 1086), the Moorfields Eye Hospital and UCL Institute of Ophthalmology Biomedical Research Center for Ophthalmology (BMRC084) and the Wellcome Trust (098481/Z/12/Z). The authors wish to thank Patrick Massae for his help with the sample collection and Athumani Rhamadhan for testing of the samples for Chlamydial infection, David Simpson for his advice on the microarray analysis and Garima Sharma for her help with the macrophage studies.
Author Contributions
Z.-Z.K. performed the experiments and analysis, and wrote the manuscript. D.G.E. contributed to the establishment of the primary fibroblast cell lines and contributed to the intellectual input of the project. M.J.B. provided the tissue samples, contributed to data analysis and project supervision, and to the editing of the manuscript. M.B. supervised the project, provided intellectual input, contributed to and edited the manuscript.

Additional Information
Supplementary information accompanies this paper at http://www.nature.com/srep

Competing financial interests: The authors declare no competing financial interests.

How to cite this article: Kechagia, J.Z. et al. Fibroblasts profiling in scarring trachoma identifies IL-6 as a functional component of a fibroblast-macrophage pro-fibrotic and pro-inflammatory feedback loop. Sci. Rep. 6, 28261; doi: 10.1038/srep28261 (2016).

This work is licensed under a Creative Commons Attribution 4.0 International License. The images or other third party material in this article are included in the article’s Creative Commons license, unless indicated otherwise in the credit line; if the material is not included under the Creative Commons license, users will need to obtain permission from the license holder to reproduce the material. To view a copy of this license, visit http://creativecommons.org/licenses/by/4.0/