Lens Epithelial Cells Initiate an Inflammatory Response Following Cataract Surgery

Jian Jiang,1,2 Mahbubul H. Shihan,2 Yan Wang,2 and Melinda K. Duncan2

1Department of Ophthalmology, Xiangya Hospital, Central South University, Changsha, Hunan, China
2Department of Biological Sciences, University of Delaware, Newark, Delaware, United States

PURPOSE. Lens epithelial cell (LEC) conversion to myofibroblast is responsible for fibrotic cataract surgery complications including posterior capsular opacification. While transforming growth factor beta (TGFβ) signaling is important, the mechanisms by which the TGFβ pathway is activated post cataract surgery (PCS) are not well understood.

METHODS. RNA-seq was performed on LECs obtained from a mouse cataract surgery model at the time of surgery and 24 hours later. Bioinformatic analysis was performed with iPPathwayGuide. Expression dynamics were determined by immunofluorescence.

RESULTS. The LEC transcriptome is massively altered by 24 hours PCS. The differentially expressed genes included those important for lens biology, and fibrotic markers. However, the most dramatic changes were in the expression of genes regulating the innate immune response, with the top three altered genes exhibiting greater than 1000-fold upregulation. Immunolocalization revealed that CXCL1, S100a9, CSF3, COX-2, CCL2, LCN2, and HMOX1 protein levels upregulate in LECs between 1 hour and 6 hours PCS and peak at 24 hours PCS, while their levels sharply attenuate by 3 days PCS. This massive upregulation of known inflammatory mediators precedes the infiltration of neutrophils into the eye at 18 hours PCS, the upregulation of canonical TGFβ signaling at 48 hours PCS, and the infiltration of macrophages at 3 days PCS.

CONCLUSIONS. These data demonstrate that LECs produce proinflammatory cytokines immediately following lens injury that could drive postsurgical flare, and suggest that inflammation may be a major player in the onset of lens-associated fibrotic disease PCS.

Keywords: posterior capsular opacification, inflammation, fibrosis, cataract surgery, lens epithelial cell

Cataracts have traditionally been the most prevalent cause of human blindness; however, in recent decades, their impact has been greatly reduced by the adoption of extracapsular and/or phacoemulsification cataract extraction followed by intraocular lens (IOL) implantation into the lens capsular bag.1–4 However, the long-term outcome of cataract surgery is compromised when residual lens epithelial cells (LECs) begin proliferating concurrently with either epithelial-mesenchymal transition (EMT) leading to the formation of profibrotic myofibroblasts, or the onset of a regenerative response where the remnant LECs convert to structurally aberrant lens fibers.5 If these LEC-derived cells remain at the periphery, they form Soemmerring’s ring, which is largely benign6 or even beneficial for long-term IOL stability.7 However, Soemmerring’s ring can continue to expand many years post cataract surgery (PCS), compromising the function of advanced IOLs,8,9 even leading to late IOL dislocation.10 If LEC-derived myofibroblasts migrate anteriorly PCS, they can cause anterior capsular fibrosis/ phimosis, which opacifies the visual axis and can decentrate the IOL.11,12 If myofibroblasts migrate onto the posterior lens capsule, they again form scar tissue in the visual axis leading to fibrotic posterior capsular opacification (PCO).13,14 Finally, even if the posterior lens capsule is ablated at the time of surgery, lens-derived myofibroblasts can opacify the visual axis by migrating from the lens capsular bag onto the anterior hyaloid membrane, particularly in pediatric patients.15,16

While there is controversy in the literature about the population-wide rates of these undesirable outcomes, PCO rates alone are reported to be 40% or higher in adult patients living 10 years or more PCS,14,17 and approach 100% in children.15,16 While these PCS side effects are generally treatable by either YAG laser ablation or surgery, poor outcomes can result due to ocular inflammation, difficulties ablating dense fibrosis, IOL displacement, and retinal complications.18–21 Thus, prevention of LEC EMT would improve the long-term visual outcome of cataract surgery.14,19

Transforming growth factor beta (TGFβ) signaling can drive LEC EMT,22 while sustained TGFβ signaling has been observed in both fibrotic PCO23 and the lens fibrotic disease, anterior subcapsular cataract (ASC).24,25 However, while TGFβ concentrations are high in the eye even prior to surgery, most of this TGFβ is in an inactive form26 and is thus unable to elicit fibrotic responses. This makes it likely that the induction of pathways that result in latent TGFβ activation27–30 are key steps in PCO pathogenesis.

We developed an in vivo mouse model of cataract surgery where the lens fiber cells are surgically removed, leaving behind the lens capsule and attached LECs.31,32 In this model, the upregulation of mRNAs encoding fibrotic markers such as α-
smooth muscle actin (α-SMA), fibronectin, and tenasin-C are detected in remnant LECs 24 hours PCS, while the first induction of these proteins is seen 48 hours PCS. Notably, though, it takes 48 hours for the first obvious upregulation of the pSMAD2/3 levels associated with TGFβ pathway activation, and up to 5 days for a maximal response. This lag between injury and TGFβ pathway activation thus makes the mouse an excellent model to study the mechanisms by which ocular trauma/surgery results in fibrotic PCO, and we have successfully used this mouse “cataract surgery” model to direct the power of mouse genetics to the study of PCO pathogenesis. Here we use RNA-seq to discover the gene expression changes that LECs undergo after cataract surgery but prior to the onset of TGFβ signaling. This analysis revealed that LECs robustly activate the innate immune response within hours of cataract surgery and supports prior speculation that postsurgical inflammation is mechanistically related to lens capsule bag fibrosis PCS.

METHODS

Animals

All animals used in this study were treated in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research and were approved by the University of Delaware Institutional Animal Care and Use Committee under protocol 1039. Mice were either obtained from Envigo/Harlan Sprague-Dawley (C57BL/6NHsd; Indianapolis, IN, USA) or were bred in house. All mice were maintained under pathogen-free conditions at the University of Delaware animal facility under a 14/10-hour light/dark cycle.

Lens Injury/Cataract Surgery Model

This animal model for PCO development has been described previously. Mice at 10 to 16 weeks old were anesthetized with ketamine/xylazine and the pupil of one eye was dilated with tropicamide and 2.5% phenylephrine hydrochloride ophthalmic solution (Akorn, Lake Forest, IL, USA). A 3-mm central corneal incision extending into the lens capsule was made with an ophthalmic knife. The lens fiber mass was separated from the lens capsule by hydrodissection with balanced salt solution, mechanically split in two, and removed from the eye. The corneal incision was closed with 10-0 nylon suture and the anterior chamber was refilled with balanced salt solution. Mice were allowed to recover for the desired time following surgery to allow for LEC responses. Mice were then again anesthetized, and the other eye was subjected to lens fiber cell removal; they were immediately killed so that the contralateral eye could serve as a time zero control. The eyes were then harvested, and processed for further analysis.

RNA Sequencing

The lens fiber cells were removed from one eye of C57BL/6NHsd mice and 24 hours later, the other eye was operated on to create a time zero control, followed by immediate euthanasia. Lens capsular bags with attached cells were isolated, and samples from five individual mice were pooled, and flash frozen on dry ice to create one 0-hour and one 24-hour PCS biological replicate. RNA was isolated using the RNeasy Mini Kit (50) from Qiagen (Cat. No./ID: 74104; Germantown, MD, USA). One microgram total RNA was processed using the Illumina TruSeq RNA Sample Prep Kit (Cat#FC-122-1001; San Diego, CA, USA) to produce sequencing libraries, which were analyzed on an Illumina HiSeq 2500 by the Genotyping and Sequencing Center, Delaware Biotechnology Institute, University of Delaware.

Sequence data were analyzed against the Mus musculus mm10 genome build (UCSC tophat version as downloaded May 2017) by the University of Delaware Bioinformatics Core Facility using a modified MAP-Rseq pipeline implemented on the BioMix computer cluster at the Center for Bioinformatics and Computational Biology/Delaware Biotechnology Institute. Pairwise differential expression analysis was performed on features with >1 counts per million (cpm) in at least two samples and statistically analyzed using the pairwise quintile-adjusted conditional maximum likelihood method exact test with a Benjamini Hochberg false discovery rate (FDR) correction run on the EdgeR BioConductor package v 3.16.5. Biologically significant differentially expressed genes (DEGs) are defined as previously described as those exhibiting statistically significant changes (FDR < 0.05) in level between 0 and 24 hours PCS, a change in mRNA level greater than 2 reads per kilobase per million (RPKM), a fold change (FC) ≥ 2, and expression levels at either time point that were 2 RPKM or greater. Pathway analysis was performed on all genes whose expression was called “present” (>1 cpm in at least two samples) with DEGs defined as those exhibiting FC ≥ 2 and FDR ≤ 0.05 using PathwayGuide (Advaita Bioinformatics, Plymouth, MI, USA). This software package uses Impact Analysis, an approach that considers both whether DEGs participating in a particular pathway (as defined by the Kyoto Encyclopedia of Genes and Genomes, KEGG) analysis performed with KEGG release 84.0/10-26, Oct 17) are overrepresented in the gene list and their directional interactions within the pathway.

Immunofluorescence

Immunofluorescence was performed to assay protein expression levels at the cellular level as described previously. Briefly, eyes were embedded in Optimun Cutting Temperature Media (Tissue Tek, Torrance, CA, USA) immediately after harvest and stored at −80°C. Frozen sections (16 μm) were obtained with a Leica CM3050 cryostat (Leica Microsystems, Buffalo Grove, IL, USA) and mounted on Color Frost plus slides (Fisher Scientific, Hampton, NH, USA). Sections were fixed in either 1:1 acetonemethanol for 15 minutes at −20°C or 4% paraformaldehyde (PFA) for 15 minutes at room temperature (RT). After washing with PBS, slides were blocked for 1 hour at RT, then incubated with primary antibody diluted in blocking buffer (see Supplementary Table S1 for specifics on the primary antibodies, blocking buffer compositions, incubation times, and dilutions used in this study). Following primary antibody treatment, slides were washed three times with PBS, then incubated for 1 hour at RT with 1:200 dilution of Alexa Fluor 488- or 568-labeled secondary antibody (Invitrogen, Grand Island, NY, USA) in PBS. DNA/cell nuclei were detected by adding fluorescein-labeled anti-DNA (Sigma-Aldrich Corp., St. Louis, MO, USA) or 1:1000 dilution of 4’,6-diamidino-2-phenylindole (DAPI; Fluoropure D21490; Thermo Fisher/Invitrogen, Carlsbad, CA, USA) to the secondary antibody solution. Some experiments also included a 1:250 dilution of fluorescein-labeled anti-α-SMA (Sigma-Aldrich Corp., St. Louis, MO, USA) in the secondary detection solution to visualize myofibroblasts. Slides were then washed with PBS three times and coveredslipped using antifade mounting media. All slides were stored at −20°C while awaiting analysis. Each staining experiment/time point was replicated using at least three independent specimens.

Confocal Imaging

Fluorescently labeled slides were visualized using a Zeiss 780 LSM confocal microscope (Carl Zeiss, Inc., Gottingen, Germany), and comparisons between images were made.
between slides imaged using identical imaging parameters. In some cases, the brightness and contrast were adjusted to allow viewing on diverse computer screens; however, these adjustments were made identically for all images within a particular time course.

RESULTS

The Lens Epithelial Cell Transcriptome Is Drastically Altered by 24 Hours Following Cataract Surgery

While it is accepted that fibrotic PCO results from the EMT of LECs driven by TGFβ signaling,5,43 we have previously shown that there is a 48-hour or longer lag between cataract surgery and the onset of robust Smad-mediated TGFβ signaling in LECs in a mouse model, likely due to the need to activate latent TGFβ PCS.33 Thus, we used RNA-seq to gain insight into the initial response of LECs to cataract surgery by comparing the transcriptome of LECs isolated immediately following surgery with that of LECs isolated 24 hours later. The resulting dataset, which includes three biological replicates from both time-zero and 24-hour PCS LECs, was submitted to the Gene Expression Omnibus (GEO) under accession number GSE111430. This analysis revealed that 14,454 genes exhibited measurable expression in LECs, while 2251 were DEGs in LECs (1255 upregulated, 996 downregulated) isolated at 24 hours PCS compared to 0 hour PCS (FDR corrected P ≤ 9.1 × 10⁻⁴), with 19 of the 27 known cataract-associated genes in the KEGG database being differentially expressed in LECs by 24 hours PCS. Notably, seven of the other nine significant predicted disease associations (FDR corrected P ≤ 0.028–0.036) are chronic autoimmune/inflammatory/infectious conditions.

Analysis of the DEGs for disease associations using iPathwayGuide (Advaita Corporation, Plymouth, MI, USA) revealed that “cataract” was the most significant (FDR corrected P = 9.1 × 10⁻⁴), with 19 of the 27 known cataract-associated genes in the KEGG database being differentially expressed in LECs by 24 hours PCS. Of these, 14 are downregulated (Sipa1H3,44 Gja3,45 Mip,46 Foxe3,47 Gja8,48 Bfsp2,50 Tdrd7,50 Maf,51 Cryab,52 Bfsp1,52 Cryaa,52 Ptx3,51 Hsf4,51 and Pax653), while 5 are upregulated (Vim,52 Wfs1,54 Epha2,55 Ftl1,56 and Gcnt257), suggesting that the “lens” phenotype of LECs is perturbed by 24 hours PCS. Notably, seven of the other nine significant predicted disease associations (FDR corrected P = 0.028–0.036) are chronic autoimmune/inflammatory/infectious conditions.

In order to predict which pathways are perturbed in LECs at 24 hours PCS, we used iPathwayGuide to perform impact analysis,41 which takes into account both the overrepresentation of genes within a pathway and whether the later genes in a pathway are significantly more perturbed than the earlier ones. This analysis predicts that 132 KEGG defined pathways are significantly affected in LECs by 24 hours PCS (Supplementary Fig. S1A) with the top 10 overrepresented pathways including cell adhesion molecules, actin cytoskeletal regulation, and numerous KEGG pathways associated with inflammatory responses (Supplementary Fig. S1B). Notably, 91 DEGs are known to be involved in cytokine/cytokine receptor pathway interactions (Fig. 1), including CXCL1, the DEG most upregulated in LECs PCS at 3866-fold (FDR corrected P = 1.6 × 10⁻⁵). Consistent with this, the genes differentially regulated in LECs at 24 hours PCS are also highly enriched for Gene Ontology (GO) terms related to immune responses, including...
Inflammation Following Cataract Surgery

While cataract surgery is very effective, its short-term outcome is hampered by the onset of ocular inflammation by 24 hours PCS, which is usually attributed to surgically induced breaks in the blood-aqueous barrier that allow for plasma protein leakage into the aqueous humor and immune cell infiltration. Since RNA-seq analysis revealed that the three genes most upregulated in LECs at 24 hours PCS were the mediators of innate immunity, CXCL1 (3866-fold), S100a9 (1505-fold), and CSF3/G-CSF (1119-fold) (Supplementary Table S2), we sought to determine their protein expression dynamics in lens capsular bags between 0 hour and 10 days PCS (Fig. 2). The expression of the chemokine CXCL1 was absent in capsular bags at 0 hour and 1 hour PCS (Figs. 2A, 2B) but was detected in LECs at 3 hours PCS (Figs. 2C, 2D). CXCL1 protein levels peaked in LECs at 24 hours PCS (Fig. 2E), sharply downregulated in capsular bags by 48 hours PCS (Fig. 2F), and remained low between 3 and 10 days PCS (Figs. 2G-J).

The proinflammatory alarmin S100a9 was not detected in capsular bags isolated at 0 or 3 hours PCS (Figs. 2K-M). S100a9 immunostaining was first detected in capsular bags at 6 hours PCS (Fig. 2N), which became more intense at 24 hours PCS (Fig. 2O). S100a9 levels sharply downregulated in capsular bags by 48 hours PCS (Fig. 2P) and remained low between 3 and 10 days PCS (Figs. 2Q-T).

There was a weak immunolocalization signal for CSF3 (granulocyte colony-stimulating factor; G-CSF; an important cytokine in neutrophil development) in LECs immediately PCS (Fig. 2U). This staining became more intense at 1 hour PCS (Fig. 2V) and continued to increase through 6 hours PCS (Figs. 2W, 2X), peaking at 24 hours PCS (Fig. 2Y). G-CSF protein levels declined by 48 hours PCS (Fig. 2Z) and were nearly undetectable between 3 and 10 days PCS (Figs. 2A-D').

In addition to the three most upregulated genes studied above, the RNA-seq data revealed that a number of other genes that function in diverse proinflammatory pathways were also upregulated in capsular bags by 24 hours PCS. PTSG2, the gene encoding the enzyme cyclooxygenase 2 (COX-2), which catalyzes a key step in prostaglandin synthesis, was 248-fold upregulated in capsular bags at 24 hours PCS. COX-2 protein was not detected in capsular bags immediately PCS (Fig. 3A); however, weak COX-2 immunostaining was detected 1 hour PCS (Fig. 3B) and continued to increase through 6 hours (Figs. 3C, 3D) PCS, peaking at 24 hours PCS (Fig. 3E). COX-2 levels decline by 48 hours PCS (Fig. 3F), and remain low, but are detectable at 3 and 4 days PCS (Figs. 3G, 3H). However, significant COX-2 immunostaining was associated with capsular bags at 5 days PCS (Fig. 3I), although these levels again decreased by 10 days PCS (Fig. 3J).

CCL2 encodes the chemokine, monocyte chemoattractant protein-1 (MCP-1), whose mRNA levels are 92-fold upregulated in lens capsular bags at 24 hours PCS. No CCL2 immunolabeling was detected in lens capsular bags either immediately or 3 to 6 hours PCS (Figs. 3K-M). Modest CCL2 immunolocalization was detected in capsular bags from 6 to 48 hours PCS (Figs. 3N-P) but its levels decreased thereafter. CCL2 protein was not detectable in capsular bags from 3 to 10 days PCS (Figs. 3Q-T).

LCN2 (neutrophil gelatinase-associated lipocalin/lipocalin2) is a multifunctional protein often upregulated in stressed tissues, particularly following injury. It has antimicrobial activity via its ability to scavenge microbial-derived siderophores, which is implicated in TGFβ-mediated LEC EMT, while also inducing the synthesis of proinflammatory cytokines by neutrophils. LCN2 mRNA levels upregulated 60-fold in LECs by 24 hours PCS. LCN2 protein was not detected in capsular bags at the time of surgery (Fig. 3U) but was found at modest levels at 1 and 3 hours PCS (Figs. 3V, 3W). LCN2 levels further increase in response to cytokine (270 of 590 genes associated with the term; FDR corrected $P = 1 \times 10^{-10}$), cytokine production (226 of 498 genes associated with the term; FDR corrected $P = 3.5 \times 10^{-20}$), and the innate immune response (186 of 447 genes associated with the term; FDR corrected $P = 3.7 \times 10^{-17}$).

Lens Epithelial Cells Upregulate Diverse Genes Involved in the Inflammatory Response Within the First 24 Hours of Cataract Surgery

FIGURE 2. PCS expression time course in LECs for the three most differentially expressed genes in this study. (A–J) CXCL1 protein expression (red) in LECs after lens fiber removal. CXCL1 protein was not detected in LECs at 0 hour (A) and 1 hour (B) PCS. CXCL1 expression is first detected at 3 hours PCS (C), and this becomes robust by 6 hours PCS (D). The highest CXCL1 expression was detected at 24 hours PCS (E) followed by dramatic downregulation by 48 hours PCS (F). Weak CXCL1 expression persisted at 3 days PCS (G) and 4 days PCS (H), while almost no CXCL1 staining was detected at 5 days PCS (I) and 10 days PCS (J). (K–T) S100a9 protein expression (red) in LECs PCS. There is little to no S100a9 expression in LECs at 0 hour (K), 1 hour (L), and 3 hours (M) PCS. S100a9 protein levels upregulated by 6 hours PCS (N), peaked around 24 hours PCS (O), then downregulated by 48 hours PCS (P). Low-level S100a9 expression is associated with capsular bags during 3 days (Q), 4 days (R), and 5 days (S) PCS, but largely disappears by 10 days PCS (T). (U–D') G-CSF protein expression (red) in LECs PCS. Weak G-CSF staining was observed in LECs at 0 hour PCS (U), and this staining upregulated gradually between 1 hour (V), 3 hours (W), and 6 hours (X) PCS. The highest level of G-CSF staining was seen at 24 hours PCS (Y), while this was attenuated at 48 hours PCS (Z). G-CSF levels are nearly undetectable at 3 days (A'), 4 days (B'), 5 days (C'), and 10 days (D') PCS. For all parts of figure, blue indicates DNA as visualized by Draq5 staining; scale bars: 100 μm; e, remnant lens epithelial cells/lens cells; lc, lens capsule.
Capsular bags at 6 hours PCS (Fig. 4X), and are maximal at 24 hours PCS (Fig. 3Y). LCN2 levels fall sharply by 48 hours PCS (Fig. 3Z, and are essentially undetectable between 3 and 10 days PCS (Figs. 3A'–D').

Heme oxygenase (HMOX1) is an enzyme that catalyzes the degradation of hemoglobin into bilirubin and carbon monoxide, which modulates innate and adaptive immunity while protecting cells from inflammation-induced oxidative stress. RNA-seq revealed that HMOX1 mRNA levels are 27-fold upregulated in lens capsular bags at 24 hours PCS compared to 0 hour PCS. No HMOX1 protein was detectable by immunolocalization in lens capsular bags between the time of surgery and 3 hours PCS (Figs. 3E'–G'). Modest HMOX1 staining was detected in lens capsular bags between 6 and 48 hours PCS (Figs. 3H'–J'), while HMOX1 staining was absent from capsular bags between 3 and 10 days PCS (Figs. 3K'–N'). These data in aggregate reveal that LECs rapidly initiate an inflammatory response after cataract surgery and/or lens wounding.

**Inflammatory Cells Are Associated With the Lens Capsular Bag Post Cataract Surgery**

As many of the genes induced in lens capsular bags at 24 hours PCS are known chemokines that can attract neutrophils to injury sites, we then determined the timing of leukocyte infiltration into the mouse eye PCS. Immunostaining of lens capsular bags PCS with CD11b (ITGAM, integrin alphaM, 9-fold upregulated in lens capsular bags at 24 hours PCS by RNA-seq), a widely accepted cell surface leukocyte marker with known roles in inflammation, revealed no leukocyte infiltration into the eye prior to 12 hours PCS (Figs. 4A–C, 4K–M), while the...
first CD11b-positive cells associated with lens capsular bags were detected at 18 hours PCS (Figs. 4D, 4N). The abundance of CD11b-positive cells increases from 18 hours to 3 days PCS, remains appreciable at 4 and 5 days PCS, and then falls to low levels by 10 days PCS. Similar results were obtained by immunostaining capsular bags with LY6G, a GPI-linked protein that is a recognized marker of granulocytes and peripheral neutrophils. Similar to CD11b, the first LY6G-positive cells did not arrive in the lens capsular bag until 18 hours PCS, although fewer cells stained overall (Supplementary Fig. S2) as would be expected since LY6G is found on a more restricted set of leukocytes.

Since CD11b immunostaining is unable to distinguish between neutrophils and macrophages, we then immunostained capsular bags with F4/80, an antibody that detects EMR1, a glycoprotein that is a very abundant and specific marker for mouse macrophages. This experiment revealed that no F4/80-positive macrophages were associated with lens capsular bags for the first 24 hours PCS (Figs. 5A–E, 5K–O) as expected since the EMR1 gene is not appreciably expressed in capsular bags right after surgery, and is not differentially expressed in 24-hour PCS capsular bags by RNA-seq (not shown). While occasional F4/80-positive cells were detected at 48 hours PCS (Figs. 5F, 5P; not shown), the first appreciable numbers of F4/80-positive cells were associated with lens capsule bags at 3 days PCS (Figs. 5G, 5Q), while they become more abundant at 4 and 5 days PCS (Figs. 5H, 5I, 5R, 5S). F4/80-positive cells are still associated with lens capsular bags at 10 days PCS (Fig. 5J, 5T).

Proinflammatory Cytokines Colocalize With the Epithelial Marker, β1-Integrin, in Lens Epithelial Cells at 24 Hours PCS, and in α-SMA-Positive Lens Cells at 48 Hours PCS, While These Molecules Generally Were Not Found at High Levels in Infiltrating Leukocytes

As most of the cytokines tested have been reported to be expressed by leukocytes, we then performed colocalization studies to confirm whether these genes were activating in LECs PCS, or whether the upregulation of these genes was simply reflecting leukocyte infiltration into the eye. Thus, we colocalized the cytokines of interest with β1-integrin, which is known to be abundant in LECs, particularly PCS, where it plays key roles in regulating the communication of lens cells with the capsule. This analysis revealed that all seven immune regulators studied, CXCL1, S100a9, G-CSF, COX-2, CCL2, LCN2, and HMOX1, showed a near-complete overlap with β1-integrin-positive LECs at 24 hours PCS (Supplementary Fig. S5). Further, double immunolabelling of 24 hours PCS lens capsular bags for the cytokines of interest and the leukocyte marker CD11b revealed that CXCL1, G-CSF, COX-2, CCL2, and LCN2 did not colocalize with CD11b-positive cells (Supplementary Fig. S4). In contrast, the alarmin S100a9 was found in both LECs and a subset of CD11b-positive cells (Supplementary Fig. S4), which would be expected as S100a9 has been reported to make up 40% of the cytoplasmic protein of circulating neutrophils.

In this cataract surgery model, the first induction of the fibrotic marker α-SMA protein is seen in remnant LECs at 48 hours PCS. Communostaining of the tested cytokines (purple) with CD11b (red) and α-SMA (green) revealed that these proinflammatory markers were generally expressed in α-SMA-positive lens cells, not CD11b-positive leukocytes at this stage (Fig. 6). The exception was S100a9, which was observed in both α-SMA-positive lens cells as well as CD11b-positive leukocytes (Fig. 6, arrows).

While six of the seven inflammatory modulators showed a peak of expression in LECs at 24 hours PCS, followed by a rapid downregulation, COX-2 showed a biphasic response, with the first upregulation seen at 24 hours followed by a rapid fall at 48 hours PCS (Figs. 3A–G, 7A–C), while the second increase started at 4 days PCS with a peak in COX-2 levels in 5 days PCS capsular bags (Figs. 3H–J, 7D–F). As this second wave of COX-2 immunostaining matches the timing of macrophage infiltration into capsular bags (Figs. 5G–J), and COX-2 has been reported to be abundant in macrophages, we performed communostaining of F4/80 (red) with COX-2 (green) (Fig. 7). As expected, the second wave of COX-2 expression corresponds with the influx of F4/80-positive macrophages at 4 days PCS; however, while some overlap between COX-2- and F4/80-positive cells is seen, the majority of COX-2 staining in capsular bags between 4 and 10 days PCS did not colocalize with F4/80 (Fig. 7).

Macrophage Influx and Upregulation of SMAD3 Phosphorylation (pSMAD3) During Fibrosis Post Cataract Surgery

Canonical (i.e., SMAD mediated) TGFβ signaling is recognized to be a major mediator of fibrotic PCO. However, TGF-β is produced in an inactive form and must be activated by tightly controlled mechanisms to elicit signaling. As macrophages have been implicated in the activation of TGF-β driving some fibrotic diseases, we compared the timing of macrophage influx into the lens capsular bag PCS with the onset of robust
SMAD3 phosphorylation in remnant lens cells PCS (Fig. 8). As we previously reported, pSMAD3 is undetectable by immunostaining in lens capsular bags prior to 24 hours PCS, while the first pSMAD3-positive nuclei are first detected in capsular bags at 48 hours PCS, although the staining is relatively weak (Figs. 7, 8). A robust upregulation of pSMAD3 staining in lens cells occurs between 48 hours and 3 days PCS, which corresponds to initial major influx of F4/80-positive macrophages into the area surrounding the capsular bag. The levels of pSMAD3 remain easily detectable in lens cells from 4 to 10 days PCS, and these cells are in close proximity to F4/80-positive macrophages (Fig. 8).

DISCUSSION

The EMT of LECs to myofibroblasts is recognized to produce the fibrotic tissue seen in ASC as well as the fibrotic sequelae of cataract surgery including Soemmering’s ring and the various forms of visual axis opacification (VAO) including anterior capsular contract/phimosis, PCO, and VAO due to growth of myofibroblasts along the anterior hyaloid membrane. There is robust experimental evidence supporting the hypothesis that canonical TGFβ signaling is both sufficient and necessary to induce LEC EMT, while the main signal transducer of the canonical TGFβ pathway (pSMAD2/3) is detected in both ASCs and fibrotic lens capsular bags, even years after surgery. However, TGFβ is produced in a latent form and must be activated to elicit signaling, and we have previously shown that there is a 48-hour lag between lens injury and the ability to detect pSMAD2/3 in LECs in a mouse cataract surgery model. This work sought to elucidate the early response of LECs to cataract surgery that sets up the conditions necessary for the onset of TGFβ signaling and LEC EMT.

Lens Epithelial Cells Rapidly Change Their Phenotype in Response to Surgical Lens Fiber Cell Removal

LEC are polarized epithelial cells with basal attachments on the lens capsule and apical interactions with the apical tips of lens fiber cells. These cells normally express many of the classical markers of an epithelium while also expressing genes more specific for lens function. Comparison of the LEC transcriptome at the time of surgery with LECs remaining in the eye for 24 hours PCS revealed that many genes known to be important for the lens phenotype exhibit altered expression. As expected for an EMT response, many regulators of lens cell fate and structure are downregulated, including Sipa1, FoxE3, Fox3, Tdrd7, Maf, Pitx3, Hsf4, FoxE3, and Pax6. However, at least five genes known to be important for lens development or physiology are upregulated PCS, including Vitr, Wfs1, Epha2, Eppa2, Fhl1, and Gcnt2. It is notable though that some of these upregulated genes are regulators or markers of mesenchymal cell fate or fibrosis in other systems, suggesting that their increased expression PCS also reflects the onset of LEC EMT. Finally, we detect the upregulation of transcripts encoding many myofibroblast markers in LECs at 24 hours PCS including α-SMA, tenascin C, TGFβI, fibronectin, transgelin, lysyl oxidase, collagen type I, and α5-integrin. As we are unable to detect the pSMAD2/3 indicative of TGFβ signaling in LECs at this time point (Fig. 8), this implies that the initial fibrotic response of LECs PCS is independent of TGFβ signaling, although it is possible that some TGFβ signaling is active but is below the threshold of our pSMAD2/3 detection assay.

Lens Epithelial Cells Remaining Behind PCS Rapidly Induce the Expression of Genes Important for the Innate Immune Response

The uninjured lens epithelium expresses few genes with known roles in the innate immune response. However, RNA-seq coupled with immunofluorescence revealed that a large number of genes involved in innate immunity, including those involved in numerous cytokine pathways, the prostaglandin synthesis pathway, and interleukins, were highly induced in LECs by 24 hours PCS. Many of the most upregulated genes encode either chemotaxtants that induce neutrophil/macrophage/microcyte migration from the circulation to injury sites.
or modulate innate immune responses as would be expected after wounding of any epithelium.

Notably, though, it appears that the details of the initial inflammatory cascade initiated by LECs may be unique to the lens. While RNA-seq experiments testing the early stages of abrasive wound healing in mouse skin are qualitatively consistent with our results in lens as mRNAs for genes involved in the cytokine response are elevated by 12 hours post wounding, remain quite high at 24 hours post wounding, and generally fall by 36 hours post wounding, none of the six genes that we highlighted for study in LECs (the top three most upregulated plus three others of biological interest) were included in the top 100 changed genes in abrasive skin wounding in mice.87 Further, the responses appear quite different quantitatively as well. For instance, while CXCL1 (the most elevated gene in LECs PCS) is also elevated after abrasive skin wounding, the response is much more mutated than in LECs while COX-2, whose mRNA is elevated 248-fold in LECs PCS) is also elevated after abrasive wound healing in mouse skin are qualitatively consistent with our results in lens as mRNAs for genes involved in the cytokine response are elevated by 12 hours post wounding, remain quite high at 24 hours post wounding, and generally fall by 36 hours post wounding, none of the six genes that we highlighted for study in LECs (the top three most upregulated plus three others of biological interest) were included in the top 100 changed genes in abrasive skin wounding in mice.87 Further, the responses appear quite different quantitatively as well. For instance, while CXCL1 (the most elevated gene in LECs PCS) is also elevated after abrasive skin wounding, the response is much more mutated than in LECs while COX-2, whose mRNA is elevated 248-fold in LECs PCS, is not altered in skin post abrasive wounding at any time tested.87 The diversity of transcriptional responses to wounding is further highlighted by a recent paper demonstrating that human oral mucosa and skin have very different responses to injury, including S100A8/A9 (which are among the top upregulated genes in injured lens epithelium, while CXCL1 and CCL2 (other top upregulated genes in injured mouse lens epithelium) do not upregulate after muscosal injury but are upregulated 48 hours and 5 days after incisional wounding of human skin.88

Notably, human LECs have been previously reported to synthesize interleukins, prostaglandins, and G-CSF in culture,89,90 while the time course of inflammatory cell arrival in

Inflammation Following Cataract Surgery

FIGURE 7. The late upregulation of COX-2 protein levels PCS observed in lens capsular bags only partially colocalizes with F4/80-positive macrophages. (A–F) COX-2 protein localization (green) alone. (G–L) F4-80 expression alone (red). (M–R) Merge between F4-80 immunodetection (red), COX-2 immunostaining (green), and nuclear staining as detected by Draq5 labeling of DNA (blue). No F4/80 or COX-2 immunostaining is seen at 0 hours (A, G, M). At 48 hours PCS, only the occasional F4-80-positive cell is detected and these do not stain strongly for COX-2 (B, H, N). At 3 days PCS, COX-2 levels are low in all cells associated with capsular bags, although F4/80-positive cell numbers are increasing (C, I, O). At 4 days PCS, COX-2 levels increase in most capsular bag cells and some colocalization of COX-2 staining in F4/80-positive cells is seen (D, J, P), a pattern that is similar at 5 days PCS (E, K, Q). By 10 days PCS, the numbers of F4/80-positive cells appear to decline along with the intensity of COX-2 immunostaining (F, L, R). Scale bars: 100 μm. c, remnant lens epithelial cells/lens cells; lc, lens capsule.

Possible Significance of Postsurgical Inflammation

We found that the upregulation of the innate immune response in LECs likely occurs rapidly PCS as the levels for all of the proinflammatory proteins tested were elevated by 6 hours PCS, preceding the arrival of neutrophils into the eye PCS by at least 12 hours, and the arrival of macrophages by 2 to 3 days. Notably, we find that inflammatory mediators upregulate at least a day prior to TGFβ signaling PCS, while it is known that eyes with active inflammation (such as in uveitis) are more prone to aggressive fibrosis PCS.94,95 Thus, it is possible that the inflammatory response seen in LECs post wounding is an initiator of PCO. Several prior studies have attempted to
determine whether aggressive prevention of postsurgical inflammation can ameliorate PCO; however, the results are equivocal.27,34,96–99 However, in each case, only a subset of the proinflammatory pathways active in PCS have been targeted, so these studies do not definitively rule out the therapeutic potential of shutting down PCS inflammation in PCO prevention.

CONCLUSIONS
The past several decades have seen numerous advances in cataract surgery techniques and IOL implants, which have yielded huge decreases in the number of people suffering from blindness or visual disability due to cataract.1,4 Despite these advances, postsurgical inflammation and ocular fibrosis derived from EMT of residual LECs are still significant barriers preventing ideal visual outcomes.9,100–102 Overall, this study provides new insights into the pathophysiology of cataract surgery side effects and implies that the LECs remaining behind following cataract surgery are signaling centers promoting PCS inflammation.

Acknowledgments
The authors thank Samuel Novo for critical reading of the manuscript.

Supported by National Eye Institute Grants EY015279 and EY028597 to MKD. Xiaobo Xia, MD, Chair, Ophthalmology, Xiangya Hospital, supported JJ’s sabbatical leave in the Duncan lab, and The Delaware INBRE program, supported by a grant from the National Institute of General Medical Science (NIGMS; P20 GM103446) and the State of Delaware, supported the RNA-seq analysis and the University of Delaware Bioimaging Facility. The authors alone are responsible for the content and writing of the paper.

Disclosure: J. Jiang, None; M.H. Shihan, None; Y. Wang, None; M.K. Duncan, None

References
Inflammation Following Cataract Surgery


