Crosstalk Between Transforming Growth Factor Beta-2 and Toll-Like Receptor 4 in the Trabecular Meshwork

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Purpose. The trabecular meshwork (TM) is involved in the outflow of aqueous humor and intraocular pressure (IOP) regulation. Regulation of the extracellular matrix (ECM) by TGFβ2 signaling pathways in the TM has been extensively studied. Recent evidence has implicated toll-like receptor 4 (TLR4) in the regulation of ECM and fibrogenesis in liver, kidney, lung, and skin. Here, we investigated the role of TGFβ2–TLR4 signaling crosstalk in the regulation of the ECM in the TM and ocular hypertension.

Methods. Cross sections of human donor eyes, primary human TM cells in culture, and dissected mouse TM rings were used to determine *Tlr4* expression in the TM. Trabecular meshwork cells in culture were treated with TGFβ2 (5 ng/mL), TLR4 inhibitor (TAK-242, 15 μM), and a TLR4 ligand (cellular fibronectin isoform [cFN]-EDA). A/J ($n = 13$), AKR/J ($n = 7$), BALBc/J ($n = 8$), C3H/HeJ ($n = 20$), and C3H/HeOuJ ($n = 10$) mice were injected intravitreally with adenovirus 5 (Ad5).hTGFβ2$^{c226s/c228s}$ in one eye, with the uninjected contralateral eye serving as a control. Conscious IOP measurements were taken using a TonoLab rebound tonometer.

Results. Toll-like receptor 4 is expressed in the human and mouse TM. Inhibition of TLR4 signaling in the presence of TGFβ2 decreases fibronectin expression. Activation of TLR4 by cFN-EDA in the presence of TGFβ2 further increases fibronectin, laminin, and collagen-1 expression, and TLR4 signaling inhibition blocks this effect. Ad5.hTGFβ2$^{c226s/c228s}$ induces ocular hypertension in wild-type mice but has no effect in *Tlr4* mutant (C3H/HeJ) mice.

Conclusions. These studies identify TGFβ2–TLR4 crosstalk as a novel pathway involved in ECM regulation in the TM and ocular hypertension. These data further explain the complex mechanisms involved in the development of glaucomatous TM damage.

Keywords: TGFβ2, TLR4, trabecular meshwork

The glaucomas are a heterogeneous group of optic neuropathies with clinical features that include cupping of the optic disc, thinning and loss of the retinal nerve fiber layer, and characteristic visual field defects.[1] A variety of risk factors have been identified for the development of glaucoma including elevated intraocular pressure (IOP), age, family history, central corneal thickness, and steroid responsiveness. Intraocular pressure is the most significant causative risk factor for both the development and progression of glaucoma. Not all ocular hypertensive individuals develop glaucoma, but lowering IOP decreases the risk for developing glaucoma[2] and decreases glaucoma progression both early[3] and late[4] in the disease.

Intraocular pressure is regulated by aqueous humor (AH) production and drainage from the eye. The trabecular meshwork (TM) is well known to be a critical tissue in AH drainage. The TM is a porous structure consisting of a series of fenestrated beams and sheets of extracellular matrix (ECM) covered with TM cells.[5,6] The ECM of the TM is important in forming an outflow pathway for AH drainage.[7,8] The TM imparts a normal resistance to AH outflow that becomes abnormally increased in glaucoma. The ECM composition of the TM plays a major role in the regulation of IOP. The ability of the TM to respond to the dynamic changes in IOP in a homeostatic state relies on the ECM remodeling capabilities of the TM.[9] Even in a resting normal state, the TM cells express matrix metalloproteinases (MMPs), tenascin C, and alpha-smooth muscle actin, all of which are typically expressed only in tissues undergoing active remodeling.[10–15] The presence of these proteins in the TM in a resting state indicates the TM may have properties that allow it to undergo transient tissue repair as part of normal maintenance, allowing proper AH drainage from the eye and IOP regulation. There is also a great deal of evidence demonstrating changes to the TM ECM in glaucoma. Increased deposition of ECM proteins in the TM, increased AH outflow resistance, and increased IOP are all associated with primary open-angle glaucoma (POAG).[16,17] The glaucomatous TM also has increased deposition of fibronectin (FN),[18,19] fine fibrillar material,[20,21] and altered glycosaminoglycan composition.[22] These data demonstrate that the ECM architecture of the TM is important in regulating AH outflow and IOP.

It is well established that AH levels of transforming growth factor-β2 (TGFβ2) are elevated in POAG patients.[23–26] We and others have shown that TGFβ2 treatment of TM cells alters the ECM composition[27–29] and induces ECM cross-linking.[30–32] The addition of TGFβ2 elevates IOP in the anterior segment perfusion organ culture models,[27,33] and overexpression of a bioactivated form of TGFβ2 in mouse eyes causes ocular

hypertension.[34–36] Transforming growth factor-β2 is known to regulate the expression of ECM proteins through the canonical SMAD pathway as well as noncanonical signaling pathways.[37–40] We have previously demonstrated that TGFβ2 signals through the canonical SMAD and non-SMAD pathways and alters the ECM in human TM cells.[30,31] We have also demonstrated that TGFβ2 signaling through the canonical SMAD pathway is essential for TGFβ2-induced ocular hypertension in mice.[36] Additionally, the cellular fibronectin isoform EDA (cFN-EDA) is present, and induced by TGFβ2, in human TM cells and tissues, and cFN-EDA protein expression is elevated in glaucomatous TM tissues.[19] In summary, these data suggest that TGFβ2 regulates the expression of ECM proteins in the TM, and the effects of TGFβ2 signaling are a major component in the development of ocular hypertension.

Toll-like receptor 4 (TLR4) is a member of the toll-like receptor family. Toll-like receptor 4 was originally identified as the specific receptor for lipopolysaccharide (LPS).[41,42] Toll-like receptor 4 can also be activated by endogenous ligands, known as DAMPs (damage-associated molecular patterns), which are generated in situ as a result of injury, cell damage, ECM remodeling, and oxidative stress.[43,44] Recent evidence has linked DAMP-activated TLR4 signaling to fibrosis and the regulation and production of ECM proteins. Damage-associated molecular patterns (cFN-EDA, low molecular weight hyaluronan, tenascin C, among others) have been shown to activate TLR4 and augment TGFβ signaling and downstream fibrotic responses in other diseases such as hepatic fibrosis, renal fibrosis, and lesional skin and lung in scleroderma patients, as well as in *Tlr4* mutant mice.[41,45–48] The role of TLR4 in fibrogenesis has been previously studied, including identification of specific single nucleotide polymorphism alleles in *TLR4* that have been associated with a delayed progression of fibrosis in liver disease and confer an overall protective effect.[49,50] Here we demonstrate that a similar TGFβ–TLR4 crosstalk is involved in the production and regulation of the ECM in the TM as well as regulation of IOP.

Materials and Methods

Human TM Cell Culture

Primary normal human TM cell strains, NTM cells (NTM1022-02, NTM115-01, NTM210-05, and NTM176-04), were isolated from normal (nonglaucomatous) donor eyes and characterized as previously described.[19,28,51] All donor tissues were obtained and managed according to the guidelines in the Declaration of Helsinki for research involving human tissue. The transformed GTM3 cell line has previously been described.[52] Cells were cultured and maintained in Dulbecco's modified Eagle's medium (DMEM; Invitrogen-Gibco Life Technologies, Grand Island, NY, USA) containing 10% fetal bovine serum (FBS; Atlas Biologicals, Fort Collins, CO, USA) and supplemented with penicillin (100 units/mL), streptomycin (0.1 mg/mL), and L-glutamine (0.292 mg/mL) (Thermo Fisher Scientific, Rockford, IL, USA).

TLR4 Inhibition and Activation

Primary NTM cells and GTM3 cells were grown to confluency and pretreated with a TLR4 selective inhibitor, TAK-242 (also known as CLI-095; InvivoGen, San Diego, CA, USA) at 15 μM for 2 hours. TAK-242 selectively inhibits the interaction between TLR4 and its adaptor molecules, TIRAP and TRAM, via the TLR4 intracellular Cys747 residue, thereby inhibiting TLR4 downstream signaling events.[53] Cells were then incubated with TGFβ2 (5 ng/mL) and/or TAK-242 (15 μM) for 24, 48, or 72 hours in serum-free medium. For TLR4 activation studies, cellular fibronectin (cFN) containing FN-EDA was isolated from human foreskin fibroblast (F2518; Sigma-Aldrich Corp., St. Louis, MO, USA) and reconstituted with sterile phosphate-buffered saline solution (PBS) to a stock concentration of 1 mg/mL. Precautions were taken to avoid repeated thaw/freezing steps. NTM cells were grown to confluency and pretreated with TAK-242 and then subsequently incubated with serum-free medium containing TGFβ2 (5 ng/mL), and/or TAK-242 (15 μM), and/or cFN-EDA (10 μg/mL), and/or LPS (100 ng/mL) for 24, 48, or 72 hours. Western blot and quantitative (q)PCR experiments were performed as described below.

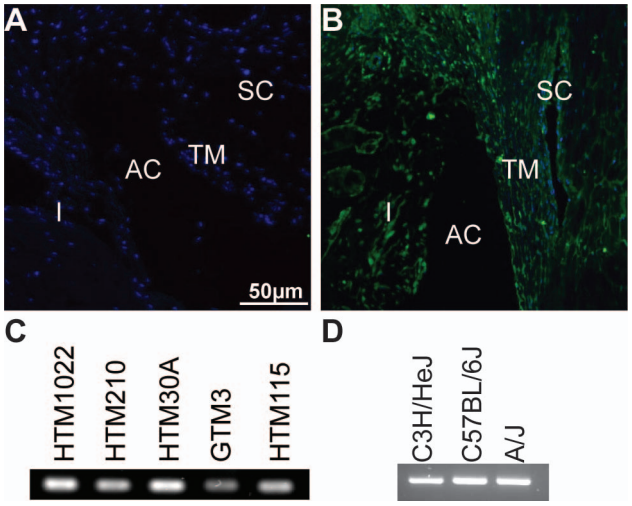

Figure 1. TLR4 is expressed in the human and mouse TM. Cross sections of eight individual human eyes were labeled with a TLR4-specific antibody. (**A**) No primary antibody control. (**B**) TLR4 expression in the human TM (TM, trabecular meshwork; SC, Schlemm's canal; AC, anterior chamber; I, iris). (**C**) RNA was isolated from primary human TM cell strains and a human transformed TM cell line, GTM3. Using primers specific to human *TLR4*, RT-PCR was performed. *TLR4* is expressed in human TM cells. (**D**) The TM ring from mice was carefully dissected and RNA isolated. Using primers specific to mouse *Tlr4*, RT-PCR was performed. *Tlr4* is expressed in the TM ring of C3H/HeJ, C57BL/6J, and A/J mice.

Cellular FN Coating

The initial analysis with cFN-EDA was done on coated surfaces. Wells from a 24-well plate were coated with 180 μL cFN-EDA (10 μg/mL) and air-dried under sterile conditions (1–2 hours). The same volume of sterile PBS was applied to control surfaces. NTM cells were seeded (5.5×10^4 cells) on cFN-EDA–coated or uncoated surfaces. Transforming growth factor-β2 (5 ng/mL) was added and cultured for 48 hours. Western blot was performed as described below with the following exceptions. Super Signal West Dura ECL Chemiluminescence Detection kit (Pierce Biotechnology, Inc., Rockford, IL, USA) was used to develop the immunolabled signals and blots were imaged using the FluorChem 8900 Image System (Alpha Innotech, San Leandro, CA, USA).

Immunocytochemistry

Primary NTM cells were seeded on 24 well-plates on coverslips and allowed to reach confluency. After completing the treatment time course of 48 (to assess FN and laminin) or 72 (to assess collagen-1) hours, cells were washed with PBS, fixed with 4% paraformaldehyde (PFA), permeabilized with 0.05% Triton X-100 in PBS, and blocked using Superblock Blocking

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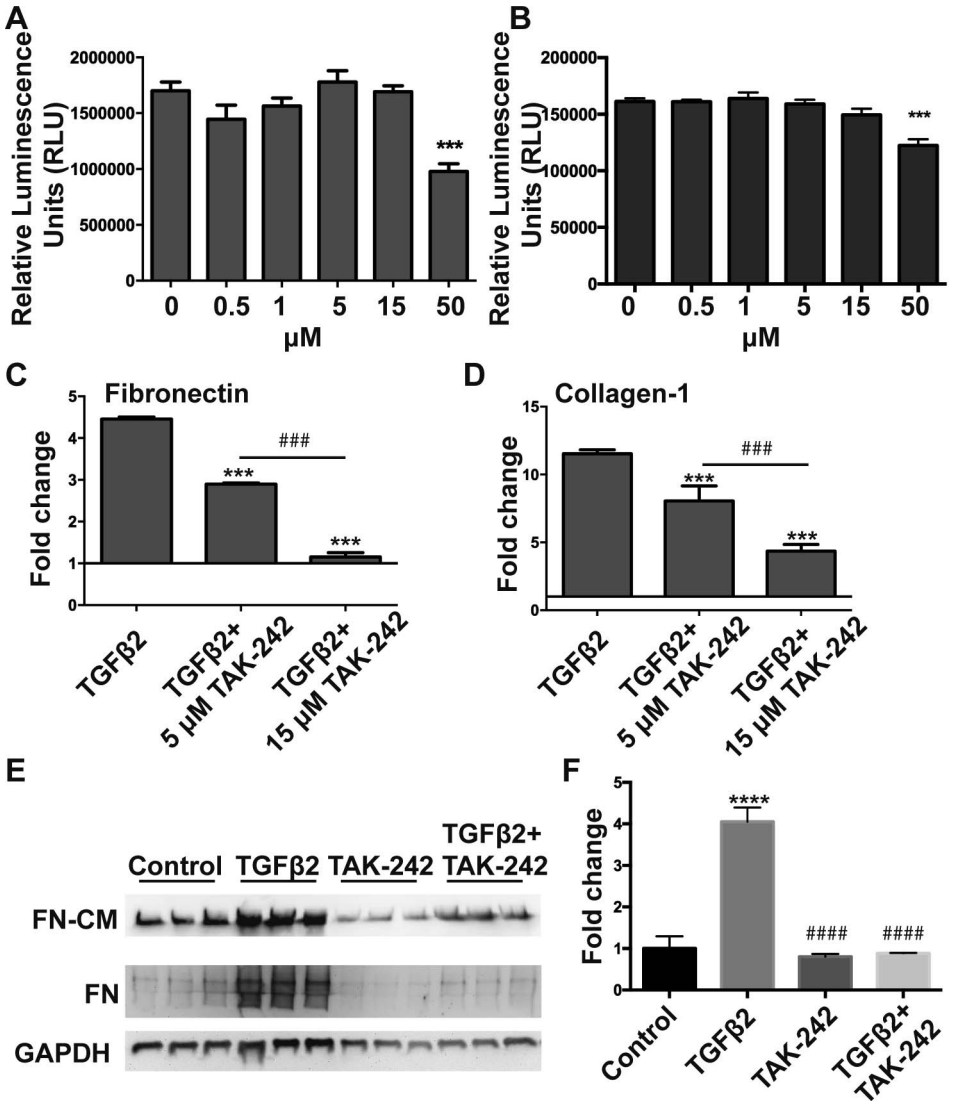

Figure 2. Inhibition of TLR4 signaling in the presence or absence of TGFβ2 decreases fibronectin and collagen-1 expression. (**A**, **B**) TM cell viability after dose response with selective TLR4 signaling inhibitor (TAK-242). The cell viability assay revealed that 50 μM TAK-242 decreased cell viability compared to untreated cells in both transformed GTM3 cells (**A**) and a primary human TM cell strain (**B**) ($n = 8$ technical replicates/cell strain). (**C**–**F**) GTM3 cells were pretreated with 5 or 15 μM TAK-242 for 2 hours, and subsequently treated with TGFβ2 for 24 hours (RNA analysis) or 48 hours (protein analysis). (**C**, **D**) TLR4 signaling inhibition decreased mRNA expression of fibronectin and collagen-1. Data represented as mean ± SEM, $n = 3$, *compared to TGFβ2-treated cells, #compared to treatment. (**E**) Western immunoblots of fibronectin-conditioned medium (FN-CM) and cell lysates. (**F**) Densitometric analysis of cell lysates shows that TLR4 signaling inhibition decreased fibronectin protein levels compared to cells treated with TGFβ2. For cell lysates, expression of fibronectin was normalized to GAPDH (loading control), and fold change was compared to control treated cells ($n = 3$). Statistical significance was determined by 1-way ANOVA and Tukey post hoc analysis, $^{***}P < 0.001$, $^{****}P < 0.0001$, $^{\#\#\#}P < 0.001$, $^{\#\#\#\#}P < 0.0001$, *compared to untreated control cells, #compared to TGFβ2-treated cells.

Buffer in PBS (Thermo Fisher Scientific) for 60 minutes at room temperature. Cells were labeled overnight at 4°C with rabbit anti-fibronectin (EMD Millipore, Billerica, MA, USA) 1:1000 dilution, anti-laminin (Novus Biologicals, Littleton, CO, USA) 1:250 dilution, and anti-collagen-1 (Novus Biologicals) 1:250 dilution in Superblock Blocking Buffer in PBS. Treatment without the primary antibody was used as a negative control. Coverslips were incubated for 2 hours using Alexa Fluor–labeled anti-rabbit (Life Technologies, Carlsbad, CA, USA) 1:1000 dilution. Coverslips were mounted to slides with Prolong Gold mounting medium containing DAPI (Invitrogen-Molecular Probes, Carlsbad, CA, USA). Image acquisition was performed using the Keyence BZ-X700 fluorescence microscope (Keyence Corporation of America; Itasca, IL, USA). All images were taken at ×200 magnification; scale bar represents 100 μm.

RT-PCR and Quantitative PCR

For the human TM cell strain and GTM3 samples, cells were washed with PBS and RNA was extracted using Isol-RNA Lysis Reagent (5PRIME, Gaithersburg, MD, USA). For the mouse samples, TM rings were carefully dissected, taking extra care to remove as much of the sclera and cornea as possible, and RNA was extracted using Isol-RNA Lysis Reagent. Samples were reverse-transcribed to cDNA (Bio-Rad iScript cDNA synthesis kit; Bio-Rad Laboratories, Hercules, CA, USA). Each PCR reaction contained: 10 μL 2× iQ SYBR Green Supermix (Bio-

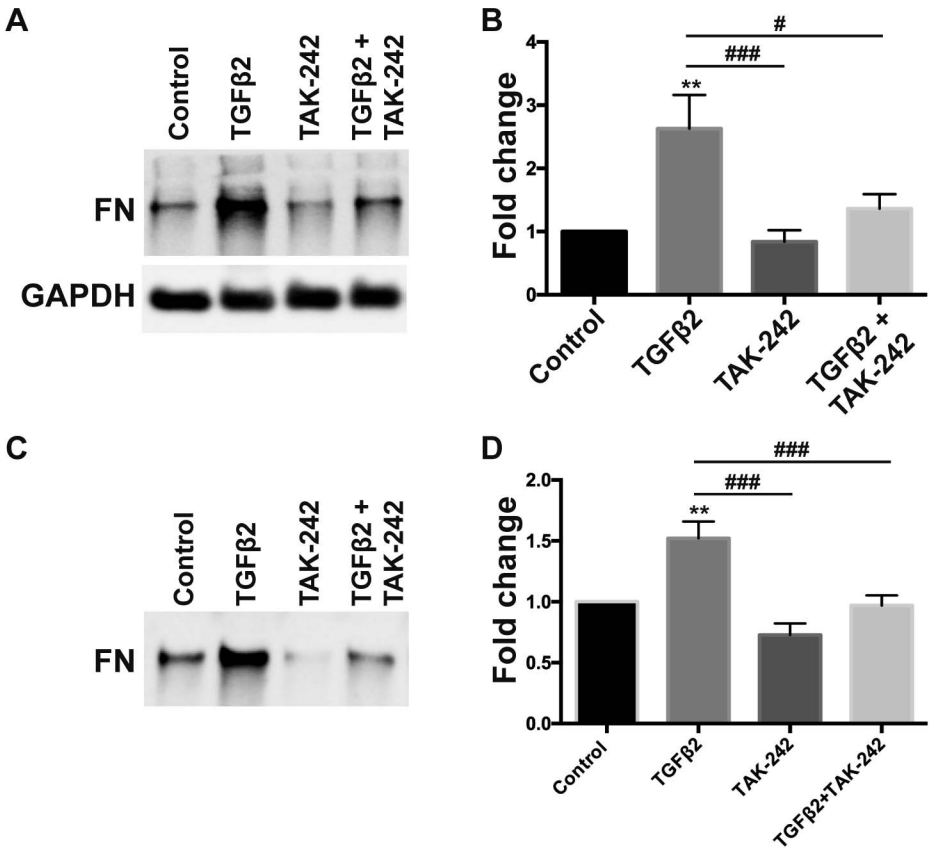

Figure 3. Inhibition of TLR4 signaling decreases TGFβ2-induced FN expression in primary TM cells. Human primary TM cells ($n = 4$ cell strains, 3 independent experiments) were pretreated with TAK-242 (15 μM) for 2 hours, and subsequently treated with TGFβ2 (5 ng/mL) for 48 hours. (**A**) Western immunoblot and (**B**) densitometric analysis of cell lysates show that TGFβ2 increases total fibronectin expression, while inhibition of TLR4 signaling with TAK-242 blocks the effect of TGFβ2. (**C**) Western immunoblot and (**D**) densitometric analysis of conditioned medium show that TGFβ2 increases total fibronectin and that inhibition of TLR4 signaling with TAK-242 blocks the effect of TGFβ2. For cell lysates, expression of fibronectin was normalized to GAPDH (loading control) and fold change was compared to control treated cells. Statistical significance was determined by 1-way ANOVA and Tukey post hoc analysis, $^{**}P < 0.01$, $\#P < 0.01$, $\#\#\#P < 0.001$ (*compared to untreated control cells, #compared to treatment).

Rad Laboratories), 0.25 μL forward primer (100 μM), 0.25 μL reverse primer (100 μM), 8.5 μL dH_2O, and 1.0 μL cDNA template (25 ng/μL). Primers used in the PCR reactions: mouse *Tlr4* (5′-AGTGGGTCAAGGAACAGAAGCA-3′, 5′-CTTTAC CAGCTCATTTCTCACC-3′),[54] human *TLR4* (5′-AGATGGGG CATATCAGAGC-3′, 5′-GTCCATCGTTTGGTTCTGG-3′),[55] *FN* (5′GGTGACACTTATGAGCGCCCTA-3′, 5′-AACATGTAGCCAC CAGTCTCAT-3′), *COL1* (5′-GGAATGAAAGG GACACAGAGG-3′, 5′-TAGCACCATCATTTCCACGA-3′), and *GAPDH* (5′-ACTC CACTCACGGCAAATTC-3′, 5′-TCTCCATGGT GGTGAAGAACA-3′). For RT-PCR experiments, samples were run on a 3% agarose gel. For qPCR, samples were run on a Bio-Rad Laboratories CFX96 Real-Time System C1000 Touch Thermal Cycler and fold change was calculated using the ΔΔCt method comparing expression to *GAPDH* and untreated control cells. Statistical significance was calculated by 1-way ANOVA and Tukey post hoc analysis.

Western Blot Analysis

All Western blot studies were performed as stated unless otherwise noted. Briefly, NTM cell strains were treated as stated above for 48 or 72 hours. Whole cell lysate and conditioned medium (CM) were collected from each condition. Cell lysates were extracted using lysis buffer (M-PER, Thermo Fisher Scientific; EDTA and protease inhibitor cocktail, Pierce Biotechnology), and Bio-Rad Dc protein Lowry assay (Bio-Rad Laboratories) was used to estimate total protein concentrations. Each loading sample contained 35 μg lysate and 4× Laemmli Buffer (Bio-Rad Laboratories), for a total volume of 40 μL. Samples were boiled for 10 minutes followed by separation using 8% SDS-PAGE. To verify equal loading for CM samples, gels were stained with Gel Code Blue Stain Reagent (Thermo Fisher Scientific). Proteins from electrophoresed gels were transferred to polyvinylidene (PVDF) membranes (Millipore, Bedford, MA, USA), and membranes were blocked with Superblock Blocking Buffer in TBS (Thermo Fisher Scientific). Membranes were immunolabeled overnight at 4°C with primary antibodies: rabbit anti-fibronectin (EMD Millipore) dilution 1:1000 and rabbit anti-GAPDH (Cell Signaling, Danvers, MA, USA) dilution 1:1000. Blots were incubated for 1 hour with horseradish peroxidase (HRP)–conjugated goat anti-rabbit secondary antibody (1:1,000; Pierce Biotechnology) diluted in Superblock Blocking Buffer in TBS. Immunolabeled signals were developed using Clarity Western ECL Substrate, and blot images were acquired using ChemiDoc Touch Imaging System (Bio-Rad Laboratories). Each experiment was repeated two or three times in each individual NTM cell strain, and a total of four independent NTM cell strains were tested. Densitometry analysis of Western immunoblot images was used to determine changes in protein content after

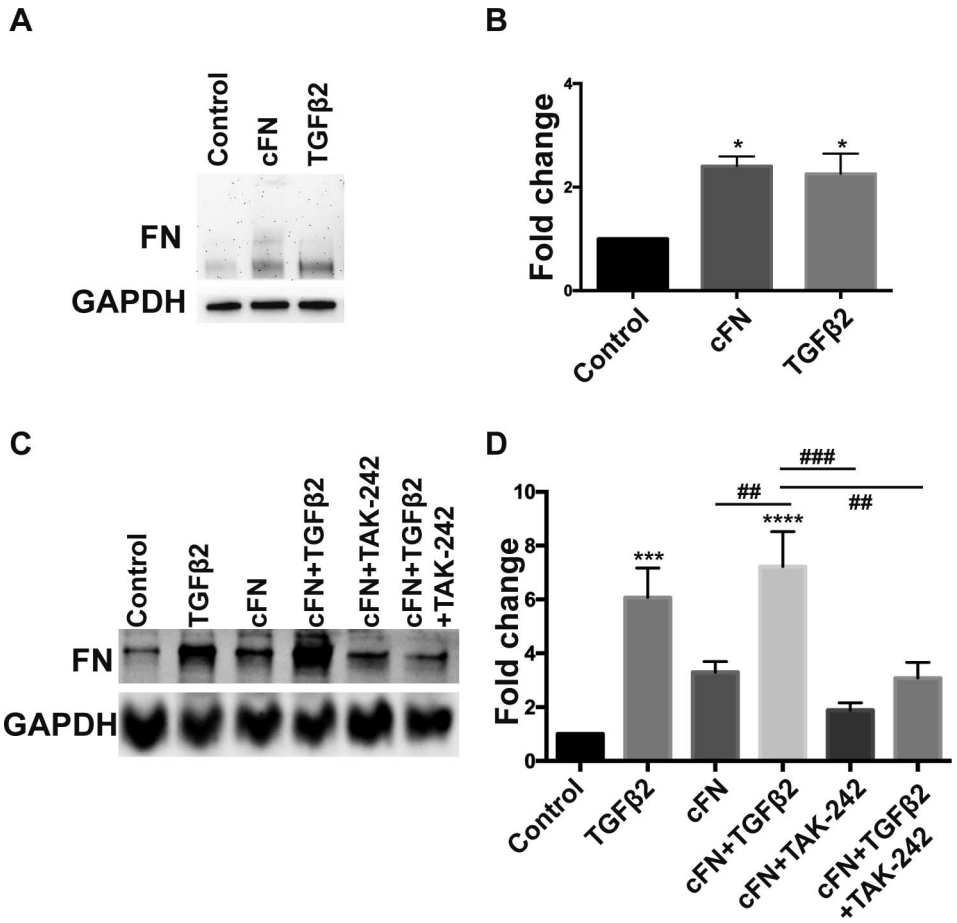

Figure 4. Activation of TLR4 by cFN containing the EDA isoform increases fibronectin expression in primary TM cells. (**A**, **B**) NTM ($n = 3$ primary human TM cells strains) were plated on cFN-EDA–coated plates and noncoated plates with TGFβ2 in serum-free medium for 48 hours. (**A**) Western immunoblot and (**B**) densitometric analysis show that cFN-EDA increases total fibronectin in cell lysates to significant comparable levels as TGFβ2. (**C**, **D**) NTM cells ($n = 4$ primary human TM cell strains, each repeated in 2 independent experiments) were pretreated with TAK-242 for 2 hours, and subsequently treated with TGFβ2 (5 ng/mL), TAK-242 (15 μM), and/or cFN-EDA for 72 hours. (**C**) Western immunoblot and (**D**) densitometric analysis show that TGFβ2 and cFN-EDA independently increase total levels of fibronectin in the cell lysates, and together amplify the total fibronectin expression. Inhibition of TLR4 signaling via TAK-242 blocks the effect of cFN-EDA and TGFβ2. Statistical significance determined by 1-way ANOVA and Tukey post hoc analysis, $^{*}P < 0.05$, $^{***}P < 0.001$, $^{****}P < 0.0001$, $\#\#P < 0.01$, $\#\#\#P < 0.001$ (*compared to control, #compared to treatment).

treatment. Band intensity for FN and GAPDH (loading control) was measured using Image Lab Software (Bio-Rad Laboratories). Each target protein densitometry value was normalized against its corresponding GAPDH value, and fold change was compared to control and represented as the mean ± SEM. Statistical significance was determined by 1-way ANOVA and Tukey post hoc analysis comparing all treatments.

Cell Viability Assay

GTM3 and primary human TM cells were plated at 5000 cells/well in a 96-well opaque walled plate with 100 μL complete medium. After 24 hours, cells were treated (8 wells/treatment). Cells were treated with TAK-242 (0.5, 1.0, 5.0, 15.0, or 50.0 μM) or vehicle control for 24 hours. Cell viability was then assessed using the CellTiter-Glo Luminescent Cell Viability Assay (Promega, Madison, WI, USA). Briefly, CellTiter-Glo Reagent was added equal to the volume of cell culture medium present (100 μL reagent to 100 μL medium containing cells). Contents were mixed for 2 minutes on an orbital shaker to induce cell lysis. The plate was incubated at room temperature for 10 minutes to stabilize luminescent signal, and luminescence was recorded.

Animals and Adenovirus Injection

All experiments were conducted in compliance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research and the University of North Texas Health Science Center (UNTHSC; Fort Worth, TX, USA) Institutional Animal Care and Use Committee (IACUC) Guidelines and Regulations. We used A/J ($n = 13$), AKR/J ($n = 7$), BALBc/J ($n = 8$), C3H/HeJ ($n = 20$), and C3H/HeOuJ ($n = 10$) mouse strains obtained from The Jackson Laboratory (Bar Harbor, ME, USA). All mice were 5 to 8 months old at the start of the experiment. All animals were housed in the UNTHSC vivarium. Adenovirus 5 (Ad5) viral vector expressing human TGFβ$^{c226s/c228s}$ (hereafter referred to as Ad5.TGFβ2) (University of Iowa, Iowa City, IA, USA) was used to overexpress TGFβ2 as previously described.[34–36] Adenovirus 5.null vector (Vector Biolabs, Malvern, PA, USA) was used as a negative control. Briefly, 2 μL of 2.5×10^7 plaque-forming units (pfu) was intravitreally injected into one eye, and the contralateral eyes were used as negative controls.

Intraocular Pressure Measurements

Intraocular pressure was measured as previously described.[34–36,56] Briefly, IOP was measured on conscious mice

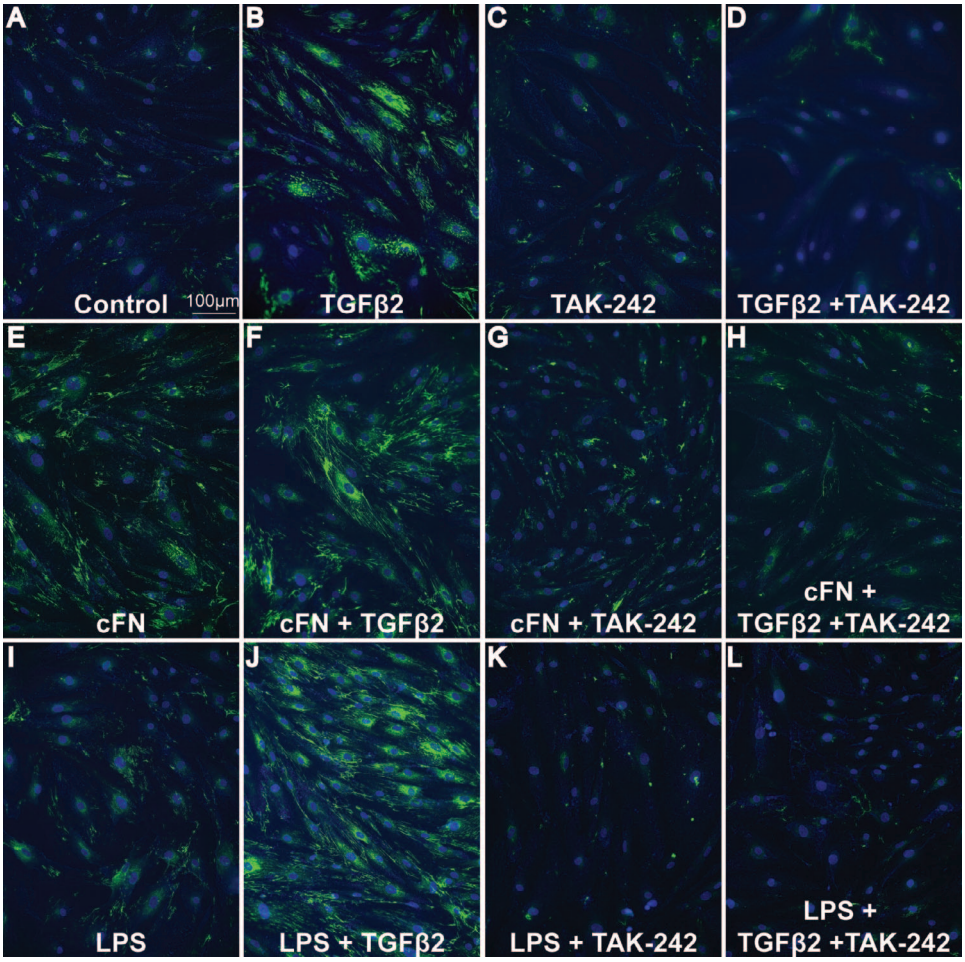

Figure 5. Effect of TGFβ2, cFN-EDA, LPS, and TLR4 signaling inhibition on FN expression in primary human TM cells. NTM cells ($n = 3$ primary human cell strains) were grown to confluency and left untreated for control (**A**), or treated with (**B**, **D**, **F**, **H**, **J**, **L**) TGFβ2 (5 ng/mL), (**E**–**H**) cFN-EDA (10 μg/mL), or (**I**–**L**) LPS (100 ng/mL). NTM cells involving TLR4 signaling inhibition were pretreated with TAK-242 (15 μM) for 2 hours (**C**, **D**, **G**, **H**, **K**, **L**), followed by treatment with (**D**, **H**, **L**) TGFβ2 (5 ng/mL), (**G**, **H**) cFN-EDA (10 μg/mL), and/or (**K**, **L**) LPS (100 ng/mL) in serum-free medium with TAK-242 (15 μM) for 48 hours. Immunocytochemistry studies revealed that TGFβ2, cFN-EDA, and LPS increased total FN expression compared to controls. Treatment with TGFβ2 and activation of TLR4 with cFN-EDA or LPS increased the staining signal when compared to TGFβ2 alone. Inhibition of TLR4 signaling via TAK-242 blocked the effect of all treatments. *Scale bar* represents 100 μm.

using the Tonolab tonometer (Colonial Medical Supply, Franconia, NH, USA). All IOP measurements were performed during the same time period of the light-on phase. Intraocular pressure exposure was calculated by subtracting the area under the curve (AUC) of the uninjected control eyes from the AUC of injected eyes for each individual animal's IOP readings over time as previously described.[35,36,56] Statistical significance was determined by Student's paired *t*-test at each time point comparing the injected eye to the contralateral uninjected control eye for A/J, BALBc/J, and AKR/J mice. Since the C3H/HeJ and C3H/HeOuJ strains were compared to each other, statistical significance was determined by 1-way ANOVA at each time point, comparing the injected eye to the contralateral uninjected control eye between each strain. All mice were 5 to 8 months old at the start of the experiment.

Immunohistochemistry of Mouse Eyes

After completion of the IOP time course, mouse eyes were enucleated and fixed in 4% PFA overnight. Eyes were embedded in paraffin, cut into 5-μm sections, and transferred to glass slides. Deparaffinization was performed by washing two times with xylene, 100% ethanol, 95% ethanol, and 50% ethanol for 2 minutes each. Slides were then soaked in PBS for 5 minutes. Tissues were blocked using Superblock Blocking Buffer in PBS (Thermo Fisher Scientific) for 30 to 60 minutes. Rabbit anti-fibronectin (EMD Millipore) 1:1000 dilution was used to label FN, followed by Alexa Fluor–labeled anti-rabbit Ig (Life Technologies) 1:1000 dilution. Prolong Gold mounting medium containing DAPI (Invitrogen-Molecular Probes) was used to mount the slides and imaged using fluorescent microscope Nikon ECLIPSE Ti-U (Nikon Instruments, Inc., Melville, NY, USA) equipped with a CRi Nuance FX Camera System (Perkin-Elmer, Waltham, MA, USA). All images were taken at ×400 magnification; scale bar represents 50 μm.

Immunohistochemistry of Human Eyes

Human donor eyes were obtained formalin fixed within 6 hours of death from regional eye banks, and were paraffin embedded and sectioned (two 5-μm sagittal sections per slide; $n = 8$ donors). Sections were deparaffinized, rehydrat-

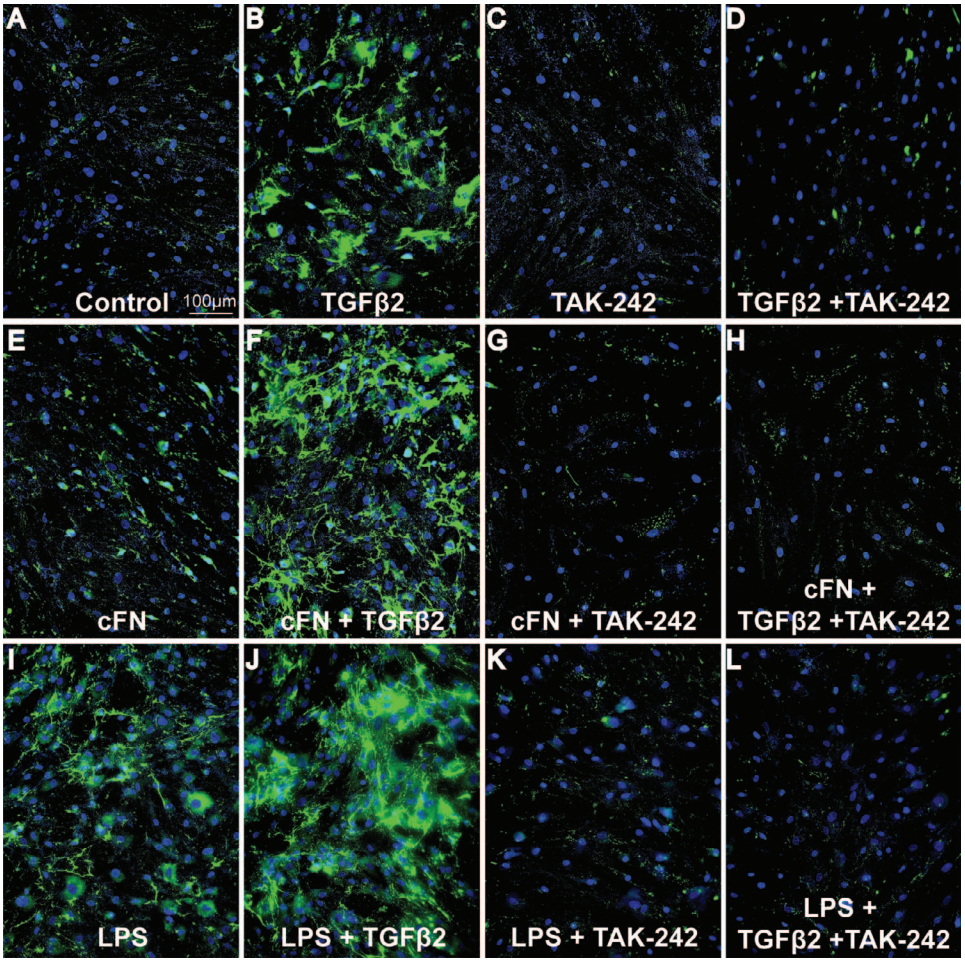

Figure 6. Effect of TGFβ2, cFN-EDA, LPS, and TLR4 signaling inhibition on COL1 expression in primary human TM cells. NTM cells ($n = 3$ primary human cell strains) were grown to confluency and left untreated for control (**A**), or treated with (**B**, **D**, **F**, **H**, **J**, **L**) TGFβ2 (5 ng/mL), (**E**–**H**) cFN-EDA (10 μg/mL), or (**I**–**L**) LPS (100 ng/mL). NTM cells involving TLR4 signaling inhibition were pretreated with TAK-242 (15 μM) for 2 hours (**C**, **D**, **G**, **H**, **K**, **L**), followed by treatment with (**D**, **H**, **L**) TGF-β2 (5 ng/mL), (**G**, **H**) cFN-EDA (10 μg/mL), and/or (**K**, **L**) LPS (100 ng/mL) in serum-free medium with TAK-242 (15 μM) for 48 hours. Immunocytochemistry studies revealed that TGFβ2, cFN-EDA, and LPS increased COL1 expression compared to controls. Treatment with TGFβ2 and activation of TLR4 with cFN-EDA or LPS increased the staining signal when compared to TGFβ2 alone. Inhibition of TLR4 signaling via TAK-242 blocked the effect of all treatments. *Scale bar* represents 100 μm.

ed, and processed for citrate/heat antigen retrieval, 15 minutes in 100°C citrate buffer (pH 6.0) followed by 15 minutes in room temperature citrate buffer (pH 6.0). Nonspecific staining was blocked by incubation for 15 minutes with 0.05 M glycine/PBS followed by 30 minutes with 5% normal goat serum/PBS. Sections were immunolabeled overnight at 4°C with rabbit anti-TLR4 antibody (1:1000) (Abcam, Cambridge, United Kingdom), washed, and incubated for an hour with secondary antibody. Secondary antibody used was donkey anti-rabbit Alexa Fluor 488 (1:500). Slides were mounted and images acquired using a Nikon Eclipse Ti inverted fluorescence microscope (Nikon, Inc.) equipped with the Cri Nuance FX Camera System (Perkin-Elmer). All images were taken at ×400 magnification; scale bar represents 50 μm.

Results

TLR4 Is Expressed in the Mouse and Human TM

Toll-like receptor 4 has been well studied in other tissues of the eye such as the retina, iris, and cornea. In addition, TLR4 was recently reported to be expressed in the TM.[57] Here we confirm these data and demonstrate that TLR4 is expressed in the human TM (Figs. 1A, 1B) and primary human TM cell cultures (Fig. 1C), as well as in the TM of several inbred mouse strains (Fig. 1D).

Inhibition of TLR4 Signaling Blocks TGFβ2-Induced ECM Production in Primary Human TM Cells

It is well established that TGFβ2 induces ECM protein production in the TM. Here, we demonstrate that inhibition of TLR4 signaling by a selective inhibitor, TAK-242, blocks the effect of TGFβ2 on ECM production. A cell viability assay and dose–response assay were performed using GTM3 cells (Fig. 2A) and a primary human cell strain (Fig. 2B) to determine the inhibitor toxicity and efficacy in TM cells. Cell viability significantly decreased at 50 μM in both GTM3 cells and the primary human TM cells (Figs. 2A, 2B). In addition, TAK-242 significantly decreased TGFβ2 (5 ng/mL)-induced FN (FN) and collagen-1 (COL1) mRNA expression at 5 and 15 μM (Figs. 2C, 2D). TAK-242 (15 μM) also significantly decreased TGFβ2-induced FN protein expression (Figs. 2E, 2F). Therefore, a 15

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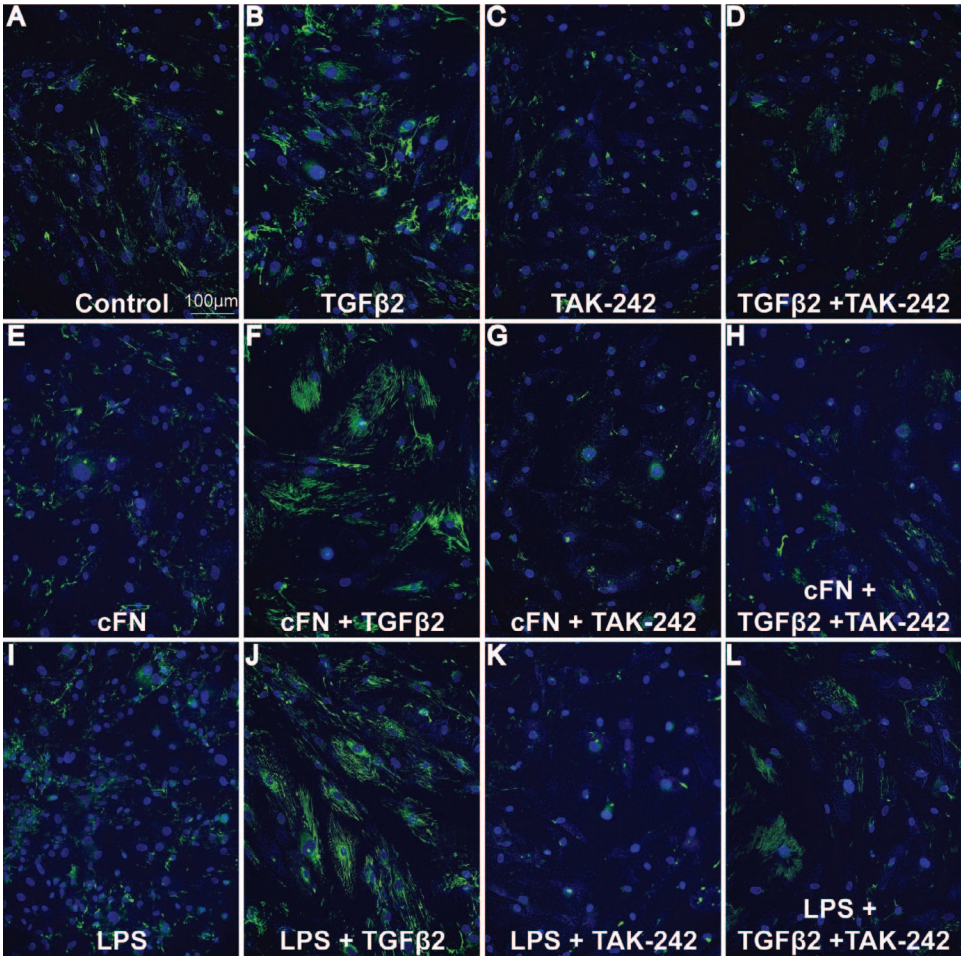

Figure 7. Effect of TGFβ2, cFN-EDA, LPS, and TLR4 signaling inhibition on laminin expression in primary human TM cells. NTM cells ($n = 3$ primary human cell strains) were grown to confluency and left untreated for control (**A**), or treated with (**B**, **D**, **F**, **H**, **J**, **L**) TGFβ2 (5 ng/mL), (**E**–**H**) cFN-EDA (10 μg/mL), or (**I**–**L**) LPS (100 ng/mL). NTM cells involving TLR4 signaling inhibition were pretreated with TAK-242 (15 μM) for 2 hours (**C**, **D**, **G**, **H**, **K**, **L**), followed by treatment with (**D**, **H**, **L**) TGFβ2 (5 ng/mL), (**G**, **H**) cFN-EDA (10 μg/mL), and/or (**K**, **L**) LPS (100 ng/mL) in serum-free medium with TAK-242 (15 μM) for 48 hours. Immunocytochemistry studies revealed that TGFβ2, cFN-EDA, and LPS increases laminin expression compared to controls. Treatment with TGFβ2 and activation of TLR4 with cFN-EDA or LPS increased the staining signal when compared to TGFβ2 alone. Inhibition of TLR4 signaling via TAK-242 blocked the effect of all treatments. *Scale bar* represents 100 μm.

μM concentration of TAK-242 was determined to be the optimal concentration for maximum inhibition of TGFβ2-induced ECM production in TM cells.

Primary TM cells were treated with TGFβ2 (5 ng/mL) and 15 μM of the TLR4 signaling inhibitor TAK-242 for 48 hours. As previously reported, TGFβ2 induced the expression of FN both in cell lysates (Figs. 3A, 3B) and in CM (Figs. 3C, 3D). However, TLR4 signaling inhibition significantly blocked TGFβ2-induced FN expression, and FN levels remained similar to control levels in both cell lysates and CM (Fig. 3) ($n = 4$ primary TM cell strains, each repeated in three independent experiments). We also demonstrated this effect at the RNA level (data not shown): TGFβ2 significantly increased FN mRNA expression 4.69- ± 0.99-fold, while TGFβ2 in the presence of TAK-242 blocked this effect with a FN mRNA expression of 1.42- ± 0.61-fold ($n = 3$ primary human TM cell strains, fold change calculated to untreated controls cells and *GAPDH* expression, $P < 0.05$ by 1-way ANOVA). There was no significant difference between untreated control cells and TGFβ2 + TAK-242–treated cells. These data suggest that the TLR4 signaling plays a major role in the TGFβ2 signaling pathway affecting the ECM in TM cells.

Activation of TLR4 Enhances TGFβ2-Induced ECM Production in Human TM Cells

Cellular FN-EDA is an isoform of FN and is a known ligand of TLR4. Previous studies have demonstrated that cFN-EDA can activate TLR4 signaling.[58] In addition, we have previously shown that cFN-EDA is significantly elevated in the glaucomatous human TM compared to normal eyes.[19] Therefore, we utilized cFN containing FN-EDA as a TLR4 activator (cFN-EDA). The role of cFN-EDA in TM cells was tested using cFN (10 μg/mL)-coated surfaces compared to uncoated control treated cells and uncoated TGFβ2-treated cells for 48 hours (Figs. 4A, 4B). Using this methodology, we demonstrate that both TGFβ2 and cFN-EDA significantly increase FN expression in cell lysates ($n = 3$ primary human TM cell strains, $P < 0.05$). These data show that cFN-EDA is able to induce the expression of FN to the same degree as TGFβ2.

We also demonstrated that cFN-EDA is able to enhance the effects of TGFβ2 in primary human TM cells by adding cFN to the culture medium. Trabecular meshwork cells were grown to confluency and then treated with cFN-EDA (10 μg/mL), TGFβ2 (5 ng/mL), and/or TAK-242 (15 μM) for 72 hours ($n = 4$ primary TM cell strains, each repeated in two independent

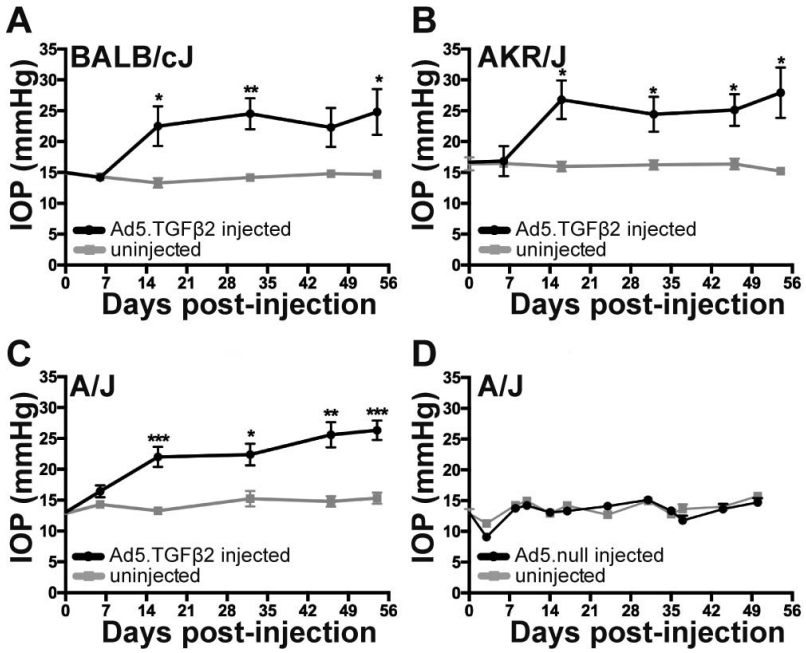

Figure 8. Ad5.TGFβ2 induces ocular hypertension in mice. Ad5.TGFβ2 (2.5×10^7 pfu) was intravitreally injected in one eye of each animal and the contralateral uninjected eyes were used as negative controls. IOP was significantly elevated in (**A**) BALBc/J ($n = 8$), (**B**) AKR/J ($n = 7$), and (**C**) A/J ($n = 13$) mice throughout an 8-week time course. (**D**) Administration of Ad5.null ($n = 5$) had no effect on IOP. These data suggest that overexpression of bioactivated TGFβ2 in the TM of mice induces ocular hypertension. Statistical significance determined by Student's paired *t*-test at each time point, $^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

experiments). As expected, TGFβ2 significantly induced FN expression compared to control cells ($P < 0.001$) (Figs. 4C, 4D). There was no significant difference between TGFβ2-treated cells and cFN-EDA-treated cells, indicating that they equally induce expression of FN in the cell lysates. Cellular FN-EDA further enhanced the TGFβ2 induction of FN protein expression (Figs. 4C, 4D). The TLR4 signaling inhibitor TAK-242 was able to block both TGFβ2- and cFN-EDA–induced expression of FN, $P < 0.01$ (Figs. 4C, 4D).

In addition to Western blotting techniques, we also tested the effect of TGFβ2 and TLR4 on ECM proteins using immunocytochemistry. Trabecular meshwork cells were grown to confluency and then treated with cFN-EDA (10 μg/mL), TGFβ2 (5 ng/mL), and/or TAK-242 (15 μM) for 48 or 72 hours. As a positive control we utilized the known TLR4 ligand, LPS (100 ng/mL), to independently activate TLR4 in our culture system. As expected, TGFβ2 induced FN (Fig. 5B), COL1 (Fig. 6B), and laminin (Fig. 7B) expression compared to untreated control cells (Figs. 5A, 6A, 7A). TAK-242 blocked the TGFβ2 induction of each of these proteins, FN (Fig. 5D), COL1 (Fig. 6D), and laminin (Fig. 7D). In addition, cFN-EDA was able to independently induce expression of FN (Fig. 5E), COL1 (Fig. 6E), and laminin (Fig. 7E). Moreover, cFN-EDA in the presence of TGFβ2 enhanced the effect of TGFβ2 alone and cFN-EDA

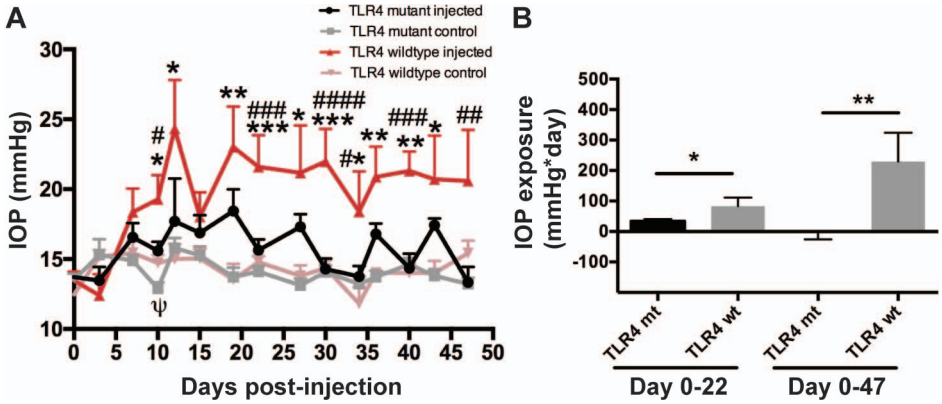

Figure 9. Ad5.TGFβ2 does not induce ocular hypertension in *Tlr4* mutant mice. Ad5.TGFβ2 (2.5×10^7 pfu) was intravitreally injected in one eye of each animal and the contralateral uninjected eyes were used as negative controls. C3H/HeJ and C3H/HeOuJ mice are genetically similar except for the genotype of *Tlr4*. (**A**) The *Tlr4* mutant strain of mice (C3H/HeJ, $n = 20$) had no biologically significant IOP elevation at any time point throughout the 6-week time course. However, Ad5.TGFβ2 induced ocular hypertension in C3H/HeOuJ ($n = 10$), which are wild type for *Tlr4*. (**B**) *Tlr4* wild-type mice had a higher IOP exposure than *Tlr4* mutant mice both early (22 days) and throughout (47 days) the time course, statistical significance determined by Student's *t*-test. For IOP measurements, statistical significance determined by 1-way ANOVA at each time point, $^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, $^{****}P < 0.0001$, $\#P < 0.05$, $\#\#P < 0.01$, $\#\#\#P < 0.001$, $\#\#\#\#P < 0.0001$. **Tlr4* wild type injected versus *Tlr4* wild-type control, *#Tlr4* wild type injected versus *Tlr4* mutant injected, Ψ = *Tlr4* mutant injected versus *Tlr4* mutant control.

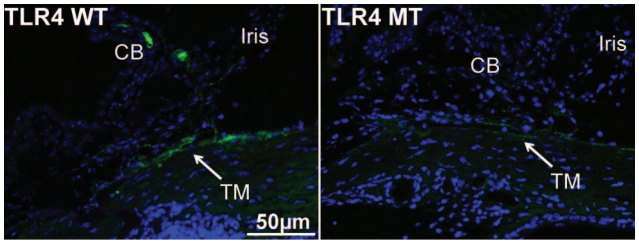

Figure 10. Fibronectin expression is increased in the TM of *Tlr4* wild-type but not *Tlr4* mutant mice treated with Ad5.TGFβ2. Mice were injected with Ad5.TGFβ2 (2.5×10^7 pfu) intravitreally in one eye of each animal and the contralateral uninjected eyes were used as negative controls. Mice were harvested 5 to 8 weeks post injection and analyzed by immunohistochemistry. (**A**) *Tlr4* wild-type mice ($n = 8$) had increased FN expression compared to (**B**) *Tlr4* mutant mice ($n = 12$). *Scale bar* represents 50 μm.

alone on FN (Fig. 5F), COL1 (Fig. 6F), and laminin (Fig. 7F). TAK-242 completely blocked the effect of both cFN-EDA and TGFβ2 on FN (Figs. 5G, 5H), COL1 (Figs. 6G, 6H), and laminin (Figs. 7G, 7H). Similar to cFN-EDA, LPS induced expression of FN (Fig. 5I), COL1 (Fig. 6I), and laminin (Fig. 7I) compared to untreated control cells, and the addition of LPS + TGFβ2 amplified the effect on FN (Fig. 5J), COL1 (Fig. 6J), and laminin (Fig. 7J). TAK-242 was also able to block the effect of both LPS and TGFβ2 on FN (Figs. 5K, 5L), COL1 (Figs. 6K, 6L), and laminin (Figs. 7K, 7L). Each experiment was repeated in three primary human TM cell strains. These data suggest a TGFβ2–TLR4 signaling crosstalk in the TM.

Mutation in TLR4 Blocks TGFβ2-Induced Ocular Hypertension in Mice

To further test the relationship between TGFβ2 and TLR4, we utilized our established mouse model of ocular hypertension using an Ad5.TGFβ2 virus containing a bioactivated form of TGFβ2.[34–36] Adenovirus 5.TGFβ2 was injected intravitreally into one eye of each animal and the contralateral uninjected eye was used as a negative control. In order to determine specific mouse strain susceptibility to Ad5.TGFβ2-induced ocular hypertension, we tested several genetically distinct inbred strains of mice. A/J ($n = 13$), BALBc/J ($n = 8$), and AKR/J ($n = 7$) all developed a significant IOP elevation for the duration of the 8-week time course in the Ad5.TGFβ2-injected eye, with no significant change in IOP in the contralateral uninjected eye (Figs. 8A–C). Adenovirus 5.null ($n = 5$) had no effect on IOP at any time point (Fig. 8D). The C3H/HeJ mouse strain has a spontaneous missense mutation in the *Tlr4* gene, which leads to a single amino acid change of a highly conserved proline to histidine at codon 712 in the cytoplasmic portion of TLR4.[41] This mutation in TLR4 impedes downstream signal transduction and produces a phenotype similar to that of *Tlr4* knockout mice.[41,42,59] Interestingly, when the C3H/HeJ mice (*Tlr4* mutant) were injected intravitreally with Ad5.TGFβ2, no biologically significant IOP elevation developed (Fig. 9A). However, the founder strain, C3H/HeOuJ (*Tlr4* wild type), which contains the wild-type *Tlr4* gene, developed significant IOP elevation after injection with Ad5.TGFβ2 (Fig. 9A), similar to what we observed in the other inbred mouse strains that also harbor a wild-type *Tlr4* allele (Fig. 8). There was no significant difference in IOP between the uninjected control eyes from C3H/HeJ and C3H/HeOuJ mice at any time point. C3H/HeJ and C3H/HeOuJ IOP data are a combination of three independent experiments (C3H/HeJ $n = 20$ [$n = 15$ days 0–47, $n = 5$ days 0–22]; C3H/HeOuJ $n = 10$ [$n = 5$ days 0–47, $n = 5$ days 0–22]). Intraocular pressure exposure was also

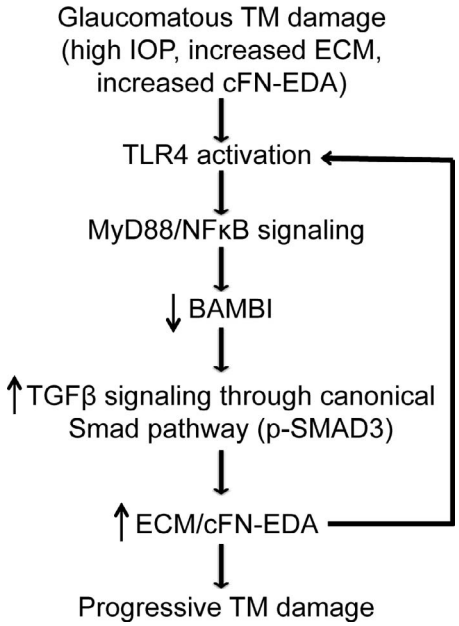

Figure 11. Proposed pathway of TGFβ2–TLR4 signaling crosstalk in the glaucomatous TM. TLR4 is activated by endogenous ligands known as DAMPs, which are produced in response to cell damage and increased ECM production and accumulation. TLR4 signals through the MyD88/NFκB signaling pathway, leading to downregulation of BAMBI and uninhibited TGFβ2 signaling. The increased TGFβ2 signaling leads to an increase in DAMP and ECM production, which can further activate TLR4. The feed-forward loop produced by TGFβ2-TLR4 signaling leads to progressive fibrosis in the TM.

calculated and *Tlr4* wild-type mice had an increased IOP exposure both early (days 0–22, $P < 0.05$) and throughout the complete time course (days 0–47, $P < 0.01$) compared to *Tlr4* mutant mice (Fig. 9B). *Tlr4* wild-type mice ($n = 13$ mice 5–8 weeks post injection) also demonstrated increased FN expression in the TM after IOP elevation compared to *Tlr4* mutant mice ($n = 7$ mice 5–8 weeks post injection) (Fig. 10). These data suggest that the TGFβ2 and TLR4 signaling pathways are involved in the development of ocular hypertension in mice.

Discussion

We present a novel pathway that is contributing to the regulation of the ECM and fibrosis in TM cells (Fig. 11). Although the ECM in TM cells is known to be important in IOP regulation, the molecular mechanisms involved in generating a glaucomatous environment in the TM remain unknown. Investigation of TGFβ2–TLR4 crosstalk in the TM further explains the mechanisms involved in the development of glaucomatous TM damage. Our data will be invaluable to the field of glaucoma and provide a framework for the development of novel therapeutic targets that could intervene and perhaps reverse glaucomatous damage to the TM.

We and others have previously published on the importance of the TGFβ signaling pathway in the regulation of ECM proteins in the glaucomatous TM and the effect on IOP.[23,25,27–31,33,34,36] In glaucoma there are vast changes to the ECM of the TM, and the disease is progressive in nature, which is similar to the changes described in other fibrotic diseases. We demonstrate that the ECM composition in the TM is regulated by crosstalk between the TGFβ signaling pathway and the TLR4 signaling pathway. Published literature supports

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these findings in other fibrotic diseased tissues such as the liver, kidney, lung, and skin.[45–48]

Upon TLR4 activation, TLR4 forms a complex with myeloid differentiation factor-2 allowing for TLR4 adaptor proteins TIRAP and TRAM to be recruited, which functions to further recruit additional adaptor proteins TRIF and MyD88.[60] Activation of the MyD88-dependent pathway ultimately leads to translocation of nuclear factor kappa B (NFκB) into the nucleus, which then functions in the regulation of many genes including cytokines, chemokines, and proteins that regulate cell cycle and cell survival, among others.[60,61] Abnormal TLR4 signaling has been linked to several inflammatory and autoimmune diseases.[62,63] In addition, DAMPs (cFN-EDA, low molecular weight hyaluronan, tenascin C, among others) have been shown to activate TLR4 and augment TGFβ signaling and downstream fibrotic responses in other diseases.[45,61,64]

Toll-like receptor 4 activation downregulates the TGFβ pseudoreceptor bone morphogenic protein (BMP) and activin membrane-bound inhibitor (BAMBI), which enhances TGFβ signaling leading to increased ECM production.[45,46] We have recently shown that BAMBI is expressed in the TM and BAMBI expression is downregulated by the presence of TGFβ2 (5 ng/mL) for 24 hours.[65] Downregulation of BAMBI by TLR4 is regulated by the MyD88-NFκB–dependent pathway.[46,61,64] BAMBI functions to inhibit TGFβ signaling by cooperating with SMAD7 and impairing SMAD3 activation, while knockdown of *Bambi* expression enhances TGFβ signaling.[66] Bone morphogenic protein and activin membrane-bound inhibitor can also interact with BMP receptors directly and antagonize BMP signaling as well as interact directly with TGFβ receptors and anatagonize TGFβ signaling.[67] Bone morphogenic proteins can suppress the TGFβ2-induced ECM deposition[28,29]; the BMP antagonist gremlin elevates IOP in perfusion-cultured anterior segments,[28] and overexpression of gremlin in mouse eyes causes ocular hypertension,[35] suggesting that BMP signaling is required for regulating normal outflow. Further experiments are needed to determine the exact mechanistic role of BAMBI in TGFβ2–TLR4 signaling in the TM; however, these data suggest that activation of TLR4 downregulates BAMBI leading to unopposed TGFβ signaling and fibrogenesis. Since the fibrotic response leads to the accumulation of endogenous TLR4 ligands such as cFN-EDA, a feed-forward loop could develop, leading to a further progression of the fibrotic response.

In summary, we demonstrate that a profibrotic TGFβ–TLR4 crosstalk is involved in the production and regulation of the ECM in the TM. These data illustrate a novel pathway involved in the development of TM damage, which could provide new targets to lower IOP and further explain the mechanisms involved in the development of glaucomatous TM damage.

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