Published in final edited form as:

Exp Eye Res. 2020 February ; 191: 107920. doi:10.1016/j.exer.2020.107920.

Nuclear Factor-Kappa Beta Signaling is Required for Transforming Growth Factor Beta-2 Induced Ocular Hypertension

Humberto Hernandez^{1,*}, Amanda L. Roberts^{1,*}, Colleen M. McDowell²

⁽¹⁾North Texas Eye Research Institute, Department of Pharmacology and Neuroscience, University of North Texas Health Science Center, Fort Worth, TX 76107

⁽²⁾University of Wisconsin-Madison, Department of Ophthalmology and Visual Sciences, Madison, WI 53706

Abstract

A major risk for the development of primary open-angle glaucoma (POAG) is elevated intraocular pressure (IOP). Elevated IOP is caused by increased outflow resistance due in part to excessive extracellular matrix (ECM) deposition in the trabecular meshwork (TM). The role of transforming growth factor beta 2 (TGFβ2) in inducing ECM production is well understood. Recent studies suggest that toll-like receptor 4 (TLR4) plays an important role in fibrogenesis. We have previously described a crosstalk between TGFβ2 and TLR4 in the development of ocular hypertension and glaucomatous TM damage. Nuclear factor-kappa beta (NF-κB) is critical for TLR4 signaling. To determine the transactivation of NF-κB, TM cells were stimulated with cellular fibronectin containing the EDA isoform (cFN-EDA), TGFβ2, or lipopolysaccharide (LPS) in combination with a selective TLR4 inhibitor. cFN-EDA, TGFβ2, and LPS all induced transactivation of NF-κB and inhibition of TLR4 blocked the effect of each treatment paradigm. To evaluate the role of NF-κB in IOP regulation, we utilized our inducible mouse model of ocular hypertension by injection of Ad5.TGFβ2 in mice harboring a mutation in NF-κB and wild-type controls. IOP was measured over time and eyes accessed by immunohistochemistry for the ECM protein FN and the specific FN-EDA isoform. Ad5.TGFβ2 induced ocular hypertension and expression of FN and FN-EDA in wild-type mice, but mutation in NF-κB blocked the effect. These data suggest that NF-κB is necessary for TGFβ2-induced ECM production and ocular hypertension and the transactivation of NF-κB is dependent on both TGFβ2 and TLR4.

Corresponding author: Colleen M. McDowell, (608)-265-3996, cmmcdowell@wisc.edu, 3375 MSC, 1300 University Ave, Madison, WI 53706.

*Contributed equally to the work

Publisher's Disclaimer: This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

Disclosure: **H. Hernandez**, None; **A.L. Roberts**, None; **C.M. McDowell**, None All authors have approved this final manuscript as being true in representation of the experimental findings.

Keywords

glaucoma; mouse model; ocular hypertension; NF- κ B; trabecular meshwork

The glaucomas are a heterogeneous group of optic neuropathies and intraocular pressure (IOP) is known to be a significant causative risk factor (Quigley, 1993). Aqueous humor (AH) production and drainage from the eye determines and regulates the IOP. The trabecular meshwork (TM) is an important tissue in AH drainage and functions to impart a normal resistance to AH outflow. The ECM composition of the TM plays a major role in the regulation of IOP and it is well known that there are significant changes to the ECM composition of the TM in glaucoma which is associated with increased AH outflow resistance and increased IOP (Lutjen-Drecoll, 1999; Rohen and Witmer, 1972). The regulation of the ECM architecture is significantly influenced by the transforming growth factor beta 2 (TGF β 2) signaling pathway. TGF β 2 levels are significantly elevated in the AH of POAG patients (Inatani et al., 2001; Ochiai and Ochiai, 2002; Ozcan et al., 2004; Tripathi et al., 1994). TGF β 2 treatment of primary and transformed TM cells alters the ECM composition and organization (Fleenor et al., 2006; Fuchshofer et al., 2007; Hernandez et al., 2017; Sethi et al., 2011; Tovar-Vidales et al., 2011; Welge-Lussen et al., 1999; Wordinger et al., 2007). Importantly, we recently demonstrated that over-expression of a bioactivated form of TGF β 2 in mouse eyes causes ocular hypertension, which is dependent on toll-like receptor 4 (TLR4) expression (Hernandez et al., 2017; Shepard et al., 2010).

TLR4 is a member of the toll-like receptor family and was originally identified as the receptor for lipopolysaccharide (LPS) (Poltorak et al., 1998). After LPS binds to TLR4, the LPS-TLR4 complex initiates signal transduction through intracellular adaptor molecules such as myeloid differentiation factor 88 (MyD88), which leads to recruitment of IL-1 receptor-associated kinases and activation of nuclear factor-kappa beta (NF- κ B) (Kawai and Akira, 2010). TLR4 can also be activated by endogenous ligands, known as damage-associated molecular patterns (DAMPs) (Miyake, 2007; Piccinini and Midwood, 2010). One such DAMP, known as fibronectin extra domain A (FN-EDA), is an isoform of FN which is upregulated as a response to tissue injury, repair, or remodeling, and during disease states (Ffrench-Constant et al., 1989; Hino et al., 1995; Kuhn et al., 1989; Muro et al., 2003). In addition, activation of TLR4 by FN-EDA has been shown to be NF- κ B dependent (Kelsh-Lasher et al., 2017; Okamura et al., 2001). DAMP activated TLR4 signaling has been linked to fibrosis and the regulation and production of ECM proteins in hepatic fibrosis, renal fibrosis, lesional skin and lung in scleroderma patients, and most importantly here to TM damage and ocular hypertension (Bhattacharyya et al., 2013; Campbell et al., 2011; Hernandez et al., 2017; Pulskens et al., 2010; Seki et al., 2007). We and others have shown that DAMPs can activate TLR4 and augment TGF β signaling and downstream fibrotic responses (Bhattacharyya et al., 2013; Guo and Friedman, 2010; Hernandez et al., 2017; Yang and Seki, 2012).

We previously demonstrated a TGF β 2–TLR4 signaling crosstalk that contributes to the development of ocular hypertension (Hernandez et al., 2017; Hernandez et al., 2018). Our *in-vitro* data show that blocking TLR4 signaling prevents the production of ECM proteins in

primary TM cells in culture (Hernandez et al., 2017). In addition, a spontaneous mutation in *Tlr4* inhibited the production of ECM proteins and prevented TGF β 2-induced ocular hypertension in mice (Hernandez et al., 2017). We also demonstrated that the DAMP, FN-EDA, enhances the TGF β 2-induced response in primary TM cells, and inhibition of TLR4 blocks this effect (Hernandez et al., 2017). The exact molecular interaction between DAMPs and TLR4 has yet to be fully elucidated. However, it is known that the DAMP, HMGB1, binds TLR4 and signals through adaptor molecules via the Toll/IL-1 receptor-domain to MyD88, IRAK, TRAF and ultimately to NF-kB (Yang et al., 2010). Similar to HMGB-1, FN-EDA has been shown to activate TLR4 leading to NF-kB activation and induction of downstream responses (Kelsh-Lasher et al., 2017; Okamura et al., 2001). Interestingly, TLR4 activation also downregulates the TGF β pseudoreceptor BMP and the activin membrane-bound inhibitor (BAMBI). BAMBI normally functions to inhibit TGF β signaling (Bhattacharyya et al., 2013; Seki et al., 2007; Yan et al., 2009). BAMBI downregulation by TLR4 is regulated by the NF-kB-dependent pathway (Guo and Friedman, 2010; Seki et al., 2007; Yang and Seki, 2012). We have also shown that knockdown of BAMBI induces ocular hypertension in mice (Hernandez et al., 2018). Together, these data demonstrate that the TLR4 signaling pathway is an important regulator of IOP homeostasis. Since TLR4 signaling ultimately leads to NF-kB activation, we hypothesized that NF-kB would be important in the development of ocular hypertension.

NF-kB resides in the cytoplasm in its inactive form and is composed by the heteromeric complex of p50, p65, and the inhibitor of the nuclear factor kappa B family of proteins (IkB alpha) (Fraczek et al., 2013; Phan et al., 2015; Strnad and Burke, 2007). Activation of TLR4 leads to proteasomal degradation of I Kappa Beta (IkB), release and exposure of the nuclear localization signal of RelA-p50 (NF-kB), and translocation to the nucleus. To test the hypothesis that NF-kB plays a role in TGF β 2-TLR4 crosstalk in the TM, transactivation studies were carried out on transformed TM cells (GTM3). The transformed GTM3 cell line has previously been described (Pang et al., 1994). Since primary TM cells are difficult to transfect (Stamer and Clark, 2017), GTM3 cells were used in this study. 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). Briefly, GTM3 were plated at a density of 1×10^5 per mL and transfected with the NF-kB luciferase reporter vector constitutively expressing *Renilla* luciferase vector or negative control vector (Quigen, Germantown, MD, USA) and the Dual-Glo Luciferase Assay System (Promega, Madison, Wisconsin, USA). Cells underwent transfection with serum free media overnight according to the manufacturer's instructions. Cells were treated with TAK-242 (CLI-095; InvivoGen, San Diego, CA, USA) at 15 μ M for 60 minutes, followed by treatment with cFN-EDA (F2518; Sigma-Aldrich Corp., St. Louis, MO, USA) or LPS (100 ng/mL) (Sigma-Aldrich Corp., St. Louis, MO, USA). Interestingly, cFN-EDA, TGF β 2, and LPS all increased the activity of NF-kB, and pretreatment with the TLR4 inhibitor, TAK-242, blocked the effect of each individual treatment (Figure 1A). No signal was detected from the negative control vector (data not shown). These data suggest that cFN-EDA, TGF β 2, and LPS activate NF-kB, NF-kB transactivation is mediated by the

TLR4 pathway, and TGF β 2 signaling likely leads to the expression of TLR4 ligands which initiate downstream transactivation of NF- κ B.

To further test the role of NF- κ B in TGF β 2-induced ocular hypertension, we employed our established mouse model of ocular hypertension using Adenovirus 5 (Ad5) viral vector expressing human TGF $\beta^{c226s/c228s}$ (hereafter referred to as Ad5.TGF β 2) (University of Iowa Vector Core, Iowa City, IA, USA) (Hernandez et al., 2017; Shepard et al., 2010). All experiments were conducted in compliance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research and approved by the University of North Texas Health Science Center Institutional Animal Care and Use Committee Guidelines and Regulations. C57BL/6J and B6.Cg-NF κ B1^{TM1Bal}/J (referred to as NF- κ B mutant mice hereafter) mouse strains were obtained from The Jackson Laboratory (Bar Harbor, ME, USA). All mice were 5 to 8 months old at the start of the experiment. Ad5.TGF β 2 was used to overexpress TGF β 2 as previously described (Hernandez et al., 2017; Shepard et al., 2010). Adenovirus 5.Null vector (Vector Biolabs, Malvern, PA, USA) was used as a negative control (Figure 1B). Briefly, 2 μ L of 2.5×10^7 plaque-forming units (pfu) was intravitreally injected into one eye, and the contralateral eyes were used as negative controls. To evaluate the role of NF- κ B on the development of ocular hypertension, intraocular measurements were performed using a rebound tonometer on isoflurane anesthetized mice as previously described (Hernandez et al., 2018). As expected, we observed that C57BL/6J wild-type mice develop ocular hypertension starting at 14 days post-injection and remain significantly elevated throughout the 42-day time course, (n=10, p<0.0001 days 14–42) (Figure 1C). However, the NF- κ B mutant mice (n=10) did not develop ocular hypertension at any time point after injection with Ad5.TGF β 2 (Figure 1C). Ad5.Null had no effect in either mouse strain (n=5 C57BL/6J, n=9 NF- κ B mutant mice) (Figure 1B). These data suggest that NF- κ B is an important regulator of TGF β 2-induced ocular hypertension.

We further analyzed the TM of Ad5.TGF β 2 injected and Ad5.null injected C57BL/6J mice and NF- κ B mutant mice at the end of the 42-day time course. We have previously demonstrated that TGF β 2 induces ECM changes to the TM both *in vivo* and *in vitro* (Hernandez et al., 2017; Hernandez et al., 2018; McDowell et al., 2015; McDowell et al., 2013). Here, we investigated whether Ad5.TGF β 2 is able to induce the expression of total FN and FN-EDA isoform in the TM of both wildtype and NF- κ B mutant mice by immunohistochemistry (Figure 2A, 2B). Eyes were enucleated and fixed in 4% paraformaldehyde for 24 hours, processed and embedded in paraffin. Five micrometer sections were cut, and sections were transferred to glass slides. Paraffin sections were dewaxed 2 times in xylene, 100% ethanol, and 95% ethanol for 2 minutes each. Slides were then soaked in PBS for 5 minutes. Sections were labeled with primary rabbit-anti-cFN-EDA antibody (1:100, NBP1–91258; Novus Biologicals) and mouse-anti-FN antibody (1:100, clone IST-4; Sigma-Aldrich), followed by secondary antibodies Alexa Fluor–labeled 488 donkey anti-rabbit Ig (1:500, Life Technologies) and Alexa Fluor-labeled 594 donkey anti-mouse Ig (1:500, Life Technologies). The mouse–anti-FN antibody recognizes epitopes on the N-terminus of FN, which are expressed by all FN isoforms. As expected, Ad5.TGF β 2 was able to induce the expression of FN-EDA isoform and total FN in C57BL/6J mice (Figure 2A). Similar to the IOP phenotype, Ad5.TGF β 2 did not induce the expression of either total FN or FN-EDA isoform in the NF- κ B mutant mice (Figure 2B). Ad5.null had no

effect on FN or FN-EDA expression in C57BL/6J mice or NF- κ B mutant mice (Figure 2C, 2D). These data suggest that NF- κ B activation may be the critical step involved in generating the TGF β 2-TLR4 induced ECM changes in the TM.

In the present study, we provide a novel role for NF- κ B in the development of ocular hypertension. We have previously described a TGF β 2-TLR4 crosstalk mechanism that plays an important role in the development of ocular hypertension. Here, we show that NF- κ B is necessary for TGF β 2-induced ECM production and ocular hypertension. Further, the transactivation of NF- κ B is dependent on both TGF β 2 and TLR4. The molecular mechanisms by which NF- κ B upregulates the ECM is not fully understood, but our data suggests it is important for development of ocular hypertension and ECM changes in the TM. These data describe a novel molecular pathway that could be used to identify innovative therapeutic targets to lower IOP and prevent glaucomatous TM damage.

Acknowledgements

The authors acknowledge Dr. Abbot Clark for many helpful discussions and technical advice. This work was supported by National Institutes of Health Grant R01EY026529 (CMM), National Institute of Health R01EY026529 R01 Diversity Supplement (CMM, ALR), National Institute of Health R25GM125587 (ALR), Neurobiology of Aging Training Grant T32AG020494 (HH), and Unrestricted grant from Research to Prevent Blindness, Inc. to the UW Madison Department of Ophthalmology and Visual Sciences.

References

- Bhattacharyya S, Kelley K, Melichian DS, Tamaki Z, Fang F, Su Y, Feng G, Pope RM, Budinger GR, Mutlu GM, Lafyatis R, Radstake T, Feghali-Bostwick C, Varga J, 2013 Toll-like receptor 4 signaling augments transforming growth factor-beta responses: a novel mechanism for maintaining and amplifying fibrosis in scleroderma. *The American journal of pathology* 182, 192–205. [PubMed: 23141927]
- Campbell MT, Hile KL, Zhang H, Asanuma H, Vanderbrink BA, Rink RR, Meldrum KK, 2011 Toll-like receptor 4: a novel signaling pathway during renal fibrogenesis. *J Surg Res* 168, e61–69. [PubMed: 20089260]
- Ffrench-Constant C, Van de Water L, Dvorak HF, Hynes RO, 1989 Reappearance of an embryonic pattern of fibronectin splicing during wound healing in the adult rat. *J Cell Biol* 109, 903–914. [PubMed: 2760116]
- Fleenor DL, Shepard AR, Hellberg PE, Jacobson N, Pang IH, Clark AF, 2006 TGFbeta2-induced changes in human trabecular meshwork: implications for intraocular pressure. *Investigative ophthalmology & visual science* 47, 226–234. [PubMed: 16384967]
- Fraczek M, Rostkowska-Nadolska B, Kapral M, Szota J, Krecicki T, Mazurek U, 2013 Microarray analysis of NF-kappaB-dependent genes in chronic rhinosinusitis with nasal polyps. *Adv Clin Exp Med* 22, 209–217. [PubMed: 23709377]
- Fuchshofer R, Yu AH, Welge-Lussen U, Tamm ER, 2007 Bone morphogenetic protein-7 is an antagonist of transforming growth factor-beta2 in human trabecular meshwork cells. *Investigative ophthalmology & visual science* 48, 715–726. [PubMed: 17251470]
- Guo J, Friedman SL, 2010 Toll-like receptor 4 signaling in liver injury and hepatic fibrogenesis. *Fibrogenesis Tissue Repair* 3, 21. [PubMed: 20964825]
- Hernandez H, Medina-Ortiz WE, Luan T, Clark AF, McDowell CM, 2017 Crosstalk Between Transforming Growth Factor Beta-2 and Toll-Like Receptor 4 in the Trabecular Meshwork. *Investigative ophthalmology & visual science* 58, 1811–1823. [PubMed: 28346614]
- Hernandez H, Millar JC, Curry SM, Clark AF, McDowell CM, 2018 BMP and Activin Membrane Bound Inhibitor Regulates the Extracellular Matrix in the Trabecular Meshwork. *Investigative ophthalmology & visual science* 59, 2154–2166. [PubMed: 29801150]

- Hino K, Shiozawa S, Kuroki Y, Ishikawa H, Shiozawa K, Sekiguchi K, Hirano H, Sakashita E, Miyashita K, Chihara K, 1995 EDA-containing fibronectin is synthesized from rheumatoid synovial fibroblast-like cells. *Arthritis and rheumatism* 38, 678–683. [PubMed: 7748223]
- Inatani M, Tanihara H, Katsuta H, Honjo M, Kido N, Honda Y, 2001 Transforming growth factor-beta 2 levels in aqueous humor of glaucomatous eyes. *Graefes Arch Clin Exp Ophthalmol* 239, 109–113. [PubMed: 11372538]
- Kawai T, Akira S, 2010 The role of pattern-recognition receptors in innate immunity: update on Toll-like receptors. *Nature immunology* 11, 373–384. [PubMed: 20404851]
- Kelsh-Lasher RM, Ambesi A, Bertram C, McKeown-Longo PJ, 2017 Integrin alpha4beta1 and TLR4 Cooperate to Induce Fibrotic Gene Expression in Response to Fibronectin's EDA Domain. *J Invest Dermatol* 137, 2505–2512. [PubMed: 28842322]
- Kuhn C 3rd, Boldt J, King TE Jr., Crouch E, Vartio T, McDonald JA, 1989 An immunohistochemical study of architectural remodeling and connective tissue synthesis in pulmonary fibrosis. *The American review of respiratory disease* 140, 1693–1703. [PubMed: 2604297]
- Lutjen-Drecoll E, 1999 Functional morphology of the trabecular meshwork in primate eyes. *Progress in retinal and eye research* 18, 91–119. [PubMed: 9920500]
- McDowell CM, Hernandez H, Mao W, Clark AF, 2015 Gremlin Induces Ocular Hypertension in Mice Through Smad3-Dependent Signaling. *Investigative ophthalmology & visual science* 56, 5485–5492. [PubMed: 26284554]
- McDowell CM, Tebow HE, Wordinger RJ, Clark AF, 2013 Smad3 is necessary for transforming growth factor-beta2 induced ocular hypertension in mice. *Experimental eye research* 116, 419–423. [PubMed: 24184030]
- Miyake K, 2007 Innate immune sensing of pathogens and danger signals by cell surface Toll-like receptors. *Semin Immunol* 19, 3–10. [PubMed: 17275324]
- Muro AF, Chauhan AK, Gajovic S, Iaconcig A, Porro F, Stanta G, Baralle FE, 2003 Regulated splicing of the fibronectin EDA exon is essential for proper skin wound healing and normal lifespan. *J Cell Biol* 162, 149–160. [PubMed: 12847088]
- Ochiai Y, Ochiai H, 2002 Higher concentration of transforming growth factor-beta in aqueous humor of glaucomatous eyes and diabetic eyes. *Jpn J Ophthalmol* 46, 249–253. [PubMed: 12063033]
- Okamura Y, Watari M, Jerud ES, Young DW, Ishizaka ST, Rose J, Chow JC, Strauss JF 3rd, 2001 The extra domain A of fibronectin activates Toll-like receptor 4. *J Biol Chem* 276, 10229–10233. [PubMed: 11150311]
- Ozcan AA, Ozdemir N, Canataroglu A, 2004 The aqueous levels of TGF-beta2 in patients with glaucoma. *Int Ophthalmol* 25, 19–22. [PubMed: 15085971]
- Pang IH, Shade DL, Clark AF, Steely HT, DeSantis L, 1994 Preliminary characterization of a transformed cell strain derived from human trabecular meshwork. *Curr Eye Res* 13, 51–63. [PubMed: 8156826]
- Phan NT, Cabot PJ, Wallwork BD, Cervin AU, Panizza BJ, 2015 Cellular and molecular mechanisms of chronic rhinosinusitis and potential therapeutic strategies: review on cytokines, nuclear factor kappa B and transforming growth factor beta. *J Laryngol Otol* 129 Suppl 3, S2–7.
- Piccinini AM, Midwood KS, 2010 DAMPening inflammation by modulating TLR signalling. *Mediators Inflamm* 2010.
- Poltorak A, He X, Smirnova I, Liu MY, Van Huffel C, Du X, Birdwell D, Alejos E, Silva M, Galanos C, Freudenberg M, Ricciardi-Castagnoli P, Layton B, Beutler B, 1998 Defective LPS signaling in C3H/HeJ and C57BL/10ScCr mice: mutations in Tlr4 gene. *Science* 282, 2085–2088. [PubMed: 9851930]
- Pulskens WP, Rampanelli E, Teske GJ, Butter LM, Claessen N, Luirink IK, van der Poll T, Florquin S, Leemans JC, 2010 TLR4 promotes fibrosis but attenuates tubular damage in progressive renal injury. *J Am Soc Nephrol* 21, 1299–1308. [PubMed: 20595685]
- Quigley HA, 1993 Open-angle glaucoma. *N Engl J Med* 328, 1097–1106. [PubMed: 8455668]
- Rohen JW, Witmer R, 1972 Electron microscopic studies on the trabecular meshwork in glaucoma simplex. *Albrecht Von Graefes Arch Klin Exp Ophthalmol* 183, 251–266. [PubMed: 4111808]
- Seki E, De Minicis S, Osterreicher CH, Kluwe J, Osawa Y, Brenner DA, Schwabe RF, 2007 TLR4 enhances TGF-beta signaling and hepatic fibrosis. *Nat Med* 13, 1324–1332. [PubMed: 17952090]

- Sethi A, Jain A, Zode GS, Wordinger RJ, Clark AF, 2011 Role of TGFbeta/Smad signaling in gremlin induction of human trabecular meshwork extracellular matrix proteins. *Investigative ophthalmology & visual science* 52, 5251–5259. [PubMed: 21642622]
- Shepard AR, Millar JC, Pang IH, Jacobson N, Wang WH, Clark AF, 2010 Adenoviral gene transfer of active human transforming growth factor- β 2 elevates intraocular pressure and reduces outflow facility in rodent eyes. *Investigative ophthalmology & visual science* 51, 2067–2076. [PubMed: 19959644]
- Stamer WD, Clark AF, 2017 The many faces of the trabecular meshwork cell. *Exp Eye Res* 158, 112–123. [PubMed: 27443500]
- Strnad J, Burke JR, 2007 IkappaB kinase inhibitors for treating autoimmune and inflammatory disorders: potential and challenges. *Trends Pharmacol Sci* 28, 142–148. [PubMed: 17287032]
- Tovar-Vidales T, Clark AF, Wordinger RJ, 2011 Transforming growth factor-beta2 utilizes the canonical Smad-signaling pathway to regulate tissue transglutaminase expression in human trabecular meshwork cells. *Experimental eye research* 93, 442–451. [PubMed: 21722634]
- Tripathi RC, Li J, Chan WF, Tripathi BJ, 1994 Aqueous humor in glaucomatous eyes contains an increased level of TGF-beta 2. *Experimental eye research* 59, 723–727. [PubMed: 7698265]
- Welge-Lussen U, May CA, Eichhorn M, Bloemendal H, Lutjen-Drecoll E, 1999 AlphaB-crystallin in the trabecular meshwork is inducible by transforming growth factor-beta. *Investigative ophthalmology & visual science* 40, 2235–2241. [PubMed: 10476788]
- Wordinger RJ, Fleenor DL, Hellberg PE, Pang IH, Tovar TO, Zode GS, Fuller JA, Clark AF, 2007 Effects of TGF-beta2, BMP-4, and gremlin in the trabecular meshwork: implications for glaucoma. *Investigative ophthalmology & visual science* 48, 1191–1200. [PubMed: 17325163]
- Yan X, Lin Z, Chen F, Zhao X, Chen H, Ning Y, Chen YG, 2009 Human BAMBI cooperates with Smad7 to inhibit transforming growth factor-beta signaling. *J Biol Chem* 284, 30097–30104. [PubMed: 19758997]
- Yang L, Seki E, 2012 Toll-like receptors in liver fibrosis: cellular crosstalk and mechanisms. *Front Physiol* 3, 138. [PubMed: 22661952]
- Yang QW, Xiang J, Zhou Y, Zhong Q, Li JC, 2010 Targeting HMGB1/TLR4 signaling as a novel approach to treatment of cerebral ischemia. *Front Biosci (Schol Ed)* 2, 1081–1091. [PubMed: 20515842]

Figure 1. NF-κB response to TLR4 and TGFβ2 signaling.

(A) Immortalized glaucomatous TM cells (GTM3) cells were transfected with 100ng of NF-κB dual-luciferase reporter (Qiagen) using Lipofectamine 3000 transfection reagent (Invitrogen). Cells were pre-treated with selective TLR4 inhibitor (TAK-242, 15μM) for 60 minutes, followed by TGFβ2 (5ng/mL), TLR4 ligand (cFN-EDA 10μg/mL) or LPS as a positive control (100ng/mL) for 24 hours. Luciferase activity was determined using the Dual-Glo Luciferase Assay System (Promega) and the Infinite M200 (Tecan). Transfection efficiency was normalized to Renilla luciferase activity. Data are represented as normalized relative light units. *** $p < 0.001$, $n = 6$ independent replicates/treatment. (B, C) NF-κB is required for Ad5.TGFβ2-induced ocular hypertension. (B) Ad5.Null had no effect on IOP in C57BL/6J mice ($n = 5$) or NF-κB mutant mice ($n = 9$). (C) Ad5.TGFβ2 induced elevated IOP starting at 14 days post-injection until day 42 post-injection in wildtype C57BL/6J mice ($n = 10$). No difference in IOP was detected at any timepoint in NF-κB mutant mice ($n = 10$). Significance was calculated by one-way ANOVA followed by Tukey post hoc analysis at each time point and p values reported are comparison of injected eye with the corresponding uninjected control eye for each treatment, **** $p < 0.0001$.

Figure 2. NF- κ B is required for Ad5.TGF β 2-induced fibronectin production

(A) Ad5.TGF β 2 induced expression of both FN and FN-EDA in the TM at 42-days post-injection in C57BL/6J mice (n=10), inset shows TM region of each image. (B). Ad5.TGF β 2 had no effect on FN or FN-EDA expression in NF- κ B mutant mice (n=10). (C, D) Ad5.null had no effect on FN or FN-EDA expression in C57BL/6J (n=5) mice or NF- κ B mutant mice (n=9).