

# Tissue plasminogen activator rescues steroid-induced outflow facility reduction via non-enzymatic action

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**Purpose:** Tissue plasminogen activator (tPA) prevents steroid-induced reduction in aqueous humor outflow facility; however, its mechanism of action at the trabecular meshwork (TM) remains unclear. Enzymatic and non-enzymatic domains allow tPA to function as both an enzyme and a cytokine. This study sought to determine whether cytokine activity is sufficient to rescue steroid-induced outflow facility reduction.

**Methods:** Outflow facility was measured in C57BL/6J mice following triamcinolone acetate exposure and either transfection of the TM using adenoviral vectors, encoding for enzymatically active and inactive tPA, or administration of the respective proteins. Protein injections were also administered to tPA deficient (*PlatKO*) and *Mmp-9* deficient (*Mmp-9KO*) mice to determine the potential to rescue reductions in outflow facility and determine downstream mechanisms. Gene expression of matrix metalloproteinases (*Mmp-2*, *-9*, and *-13*) was measured in angle ring tissues containing the TM.

**Results:** Enzymatically active and inactive tPA (either produced after TM transfection or after direct administration) were equally effective in attenuating steroid-induced outflow facility reduction in C57BL/6J mice. They were also equally effective in rescuing outflow reduction in *PlatKO* mice and causing enhanced expression of matrix metalloproteinases. However, both enzymatically active and enzymatically inactive tPA did not improve outflow reduction in *Mmp-9KO* mice or increase the baseline outflow facility in naïve C57BL/6J mice.

**Conclusions:** tPA enzymatic activity is not necessary in the regulation of aqueous humor outflow. tPA can increase the expression of matrix metalloproteinases in a cytokine-mediated fashion. This cascade of events may eventually lead to extracellular matrix remodeling at the TM, which reverses outflow facility reduction caused by steroids.

Iatrogenic steroid-induced glaucoma was first described in the 1950s [1-3]. It was later discovered that a third of the general population are moderate responders who develop intraocular pressure (IOP) elevation (6–15 mmHg) following corticosteroid exposure [4]. Steroid-induced IOP elevation is caused by a decrease in aqueous humor outflow facility [5-8]. Although the exact mechanism remains unclear, increased extracellular matrix (ECM) deposition in the trabecular meshwork (TM) has been reported and is consistently detected in both glaucomatous human specimens and animal models of the disease [5-17].

The ECM structure of the conventional outflow pathway is dynamic and continually remodeled by matrix metalloproteinases (MMPs) [1,18-20]. MMPs are zinc endopeptidases secreted in their zymogen (pro-MMP) form for subsequent activation via proteolytic cleavage [18,21]. The expression and activity of MMP-2, MMP-9, and MMP-13 are reduced in cases of primary open angle glaucoma and in animal models of ocular hypertension [22-24]. Conversely, administration

of exogenous MMPs during anterior segment organ culture perfusion experiments increases outflow facility [25].

MMP regulation occurs via cytokine-dependent transcriptional control and proteolytic post-translational activation [18]. Tissue plasminogen activator (tPA), which plays a critical role in fine-tuning both pathways of MMP activity regulation, is a serine protease best known for its role in catalyzing the conversion of plasminogen to plasmin in the fibrinolytic pathway [26]. It is comprised of catalytic/enzymatic and non-catalytic/non-enzymatic regions [26]. tPA is expressed and secreted by TM cells under physiologic conditions [26-28]. The proteolytic action of tPA allows it to activate pro-MMPs, either through plasmin activation [29] or through direct cleavage [30,31].

tPA also functions as a cytokine by promoting intracellular signaling cascades and gene expression changes following interactions with cell surface receptors, such as low-density-lipoprotein receptor-related protein 1 (LRP-1) and N-methyl-D-aspartate receptor (NMDAR) [32-36]. Through this mechanism, tPA enhances MMP transcriptional expression in brain, retinal, lung, and renal tissues [32-34,37].

The proteolytic action of tPA is dependent on the presence of serine-478 [38] at its catalytic active site. A change of serine-478 to alanine (S478A) results in a complete loss of

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tPA enzymatic activity [39,40] without affecting its binding properties [41] to receptors and inhibitors, allowing it to continue to function in a non-enzymatic fashion.

Previous studies have found that steroids cause a reduction in tPA at the TM [7,8,42], and that exogenous administration of tPA can prevent and reduce steroid-induced IOP elevation in sheep [19,20] and prevent steroid-induced reduction of outflow facility in mice [43]. On the other hand, deletion of the gene encoding tPA (*Plat*) in mice causes a significant reduction in outflow facility [44]. This effect is associated with a reduction in *Mmp-9* expression in the angle ring tissues of tPA-deficient mice [44]. Furthermore, overexpression of tPA in steroid-treated mice results in increased expression of *Mmp-2*, *Mmp-9*, and *Mmp-13* in angle ring tissue [43].

To determine whether tPA regulates aqueous humor outflow at the level of the TM via proteolytic action, cytokine action, or both, native tPA and mutant non-enzymatically active tPA (NE-tPA/S478A-tPA) were used in a mouse model of steroid-induced glaucoma, in animals under baseline conditions, in *Plat*KO mice, and in *Mmp-9*KO mice. The effect of tPA on outflow facility and *Mmp* expression was explored in these animals.

## METHODS

**Animals:** Female mice aged 8–12 weeks were used for this study. The animals were housed and bred at the State University of New York (SUNY) Downstate Health Sciences University Division of Comparative Medicine (Brooklyn, NY) under a 12 h:12 h light-dark cycle and fed ad libitum. C57BL/6J mice were obtained from The Jackson Laboratories (Bar Harbor, ME). A *Plat*KO mouse colony was established from animals (stock No. 002508) obtained from The Jackson Laboratories [45]. These animals were on a C57BL/6J background. An *Mmp-9*KO mouse colony was established from animals (stock No. 007084) obtained from The Jackson Laboratories [46]. These animals are on a mixed background, but have been bred into the C57BL/6J background for five generations. Protocols were approved by the SUNY Downstate Institutional Animal Care and Use Committee, and experiments were performed according to the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research.

**Adenoviral vector construction:** The pShuttle-CMV-PLAT construct used was previously reported [43] and contains the full coding region of the sheep *PLAT* mRNA (1.8 kb) downstream of a CMV promoter and adjacent to a human histone 2B (H2B) tagged fluorescent reporter (mCherry) gene with an internal ribosome entry site (IRES) at the multiple cloning site of the shuttle vector [43]. To generate the non-enzymatic pShuttle-CMV-PLATNE construct, the

*PLAT* gene insert was removed from the pShuttle-CMV-PLAT plasmid by restriction enzyme digestion and ligated into a pUC18 plasmid vector. Conversion of thymidine 1677 to guanosine (1677T>G) in the tPA enzyme active site was performed using the QuikChange Lightning Multi Site Directed Mutagenesis Kit, according to the manufacturer's instructions (Agilent, Santa Clara, CA). This caused alanine (GCG) to replace serine (TCG) at the catalytic site (S478A), rendering the resulting protein enzymatically inactive. The PLAT-NE construct was excised from the pUC18 vector and re-ligated into the pShuttle-CMV plasmid. All plasmids underwent restriction enzyme digestion to confirm the proper fragment sizes and orientation. The inserts were directly sequenced to confirm the nucleotide sequence (GENEWIZ, South Plainfield, NJ).

The loss of enzymatic activity in pShuttle-CMV-PLATNE was tested in comparison to pShuttle-CMV-PLAT via plasmid transfection into 80% confluent human microvascular endothelial cells (HMVEC; ThermoFisher Scientific, Waltham, MA) in a 12-well plate through the use of Targefect-RAW (Targeting Systems, El Cajon, CA). Transfection efficiency was visualized by the intracellular fluorescent expression of mCherry. Supernatants were collected from all culture plate wells, and enzymatic activity was assessed at 3 h via a Tissue type Plasminogen Activator Human Chromogenic Activity Assay Kit, per manufacturer's instructions (ab108905, Abcam Co, England). Activity was normalized for total protein amount as determined by a microBCA protein assay kit, per the manufacturer's instructions (Pierce, ThermoFisher Scientific), and expressed as a percentage of tPA activity in non-transfected cells.

An adenovirus vector carrying the non-enzymatic transgene (AdPLATNE) was generated by homologous recombination, amplification, and purification by ViraQuest Inc. (North Liberty, IA). Frozen stocks of AdPLAT and AdNull (containing no transgene) that were previously reported [43] were also used.

**Steroid, adenoviral, and protein injections:** Intracameral adenovirus and intravitreal protein injections were performed using a 10 µl Hamilton syringe with 36-gauge stainless steel needles (WPI Inc., Sarasota, FL), while subconjunctival injections were performed using a 100 µl Hamilton Syringe with 26-gauge needles (Precision Glide, Becton Dickinson & CO, Franklin Lakes, NJ). All injections were performed under isoflurane inhalation anesthesia and topical anesthesia with 0.5% proparacaine. C57BL/6J mice undergoing adenovirus treatment received bilateral injections with 20 µl of triamcinolone acetonide (TA) suspension (40 mg/ml, Kenalog-40; Bristol-Myers Squibb, New York, NY) subconjunctivally,

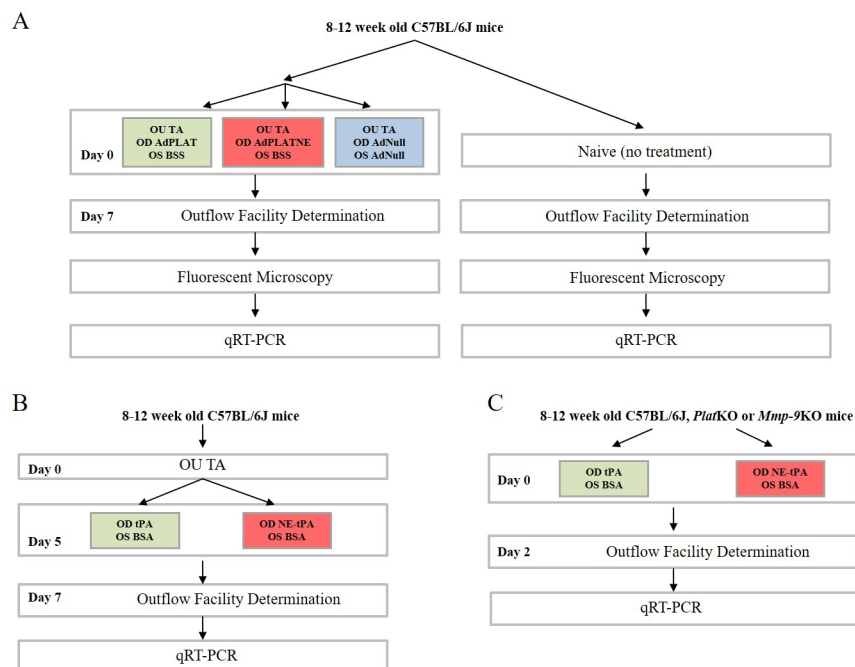


Figure 1. Experimental design outline. **A**: Animals received bilateral (oculus uterque/OU) subconjunctival injections of triamcinolone acetate (TA) and were treated with Adenoviral vectors – AdPLAT, AdPLATNE, or AdNull – in the right eye (oculus dexter/OD) and control balanced salt solution (BSS) in the left eye (oculus sinister/OS). **B** and **C**: Animals were treated with intravitreal protein (tPA or NE-tPA) injections in the right eye (OD) and control bovine serum albumin (BSA) in the left eye (OS). Naïve C57BL/6J animals were also used for comparison purposes.

immediately before the intracameral adenovirus injection. Animals were then divided into three groups (Figure 1A):

1) Animals that received unilateral intracameral injection with 2  $\mu$ l of AdPLAT suspension ( $1.1 \times 10^{12}$  vg/ml) while the contralateral eye received 2  $\mu$ l of balanced salt solution (BSS; Alcon Laboratories Inc., Fort Worth, TX),

2) Animals that received unilateral intracameral injection with 2  $\mu$ l of AdPLATNE suspension ( $1.2 \times 10^{12}$  vg/ml) while the contralateral eye received 2  $\mu$ l of BSS, and

3) Animals that received bilateral intracameral injection with 2  $\mu$ l of AdNull suspension ( $1.1 \times 10^{12}$  vg/ml).

Animals were euthanized one week after AdPLAT, AdPLATNE, or AdNull treatment via isoflurane inhalation (5%) with subsequent cervical dislocation.

C57BL/6J, *PlatKO* and *Mmp-9KO* mice were treated with intravitreal tPA (either enzymatically active or enzymatically inactive) or bovine serum albumin (BSA). C57BL/6J mice were divided into two groups:

1) Animals receiving bilateral steroid (TA) injection subconjunctivally five days before protein injection (Figure 1B), and

2) Animals not receiving subconjunctival steroids (Figure 1C).

*PlatKO* and *Mmp-9KO* mice were also not given steroids before the protein injections (Figure 1C). Within each cohort, animals were divided into two groups:

1) Animals that received unilateral intravitreal injection with 2  $\mu$ l of tPA (5  $\mu$ g/ $\mu$ l, Actilyse; Boehringer Ingelheim, Ingelheim am Rhein, Germany), while the contralateral eye received 2  $\mu$ l of BSA (5  $\mu$ g/ $\mu$ l; Gold Biotechnology, St Louis, MO), and

2) Animals that received unilateral intravitreal injections with 2  $\mu$ l of non-enzymatically active tPA (NE-tPA/S478A-tPA; 5  $\mu$ g/ $\mu$ l; Innovative Research, Novi, MI), while the contralateral eye received 2  $\mu$ l of BSA. Animals were euthanized two days after intravitreal injection.

**IOP measurement:** IOP was measured pre-terminally in *Mmp-9KO* mice with a rebound tonometer [47]. Animals were held in a custom-made restrainer that did not compress the chest or neck while IOP was measured [48]. IOP measurements were performed after the application of 0.5% proparacaine topical anesthesia. Five measurements were obtained per eye and averaged. IOP measurements were performed between 10 AM and 12 PM to minimize the effect of diurnal IOP variation.

**Outflow facility determination:** Mouse eyes were enucleated immediately after euthanasia. Outflow facility was determined using a constant pressure method, as previously described [44]. Pressure was raised in steps of 4 cmH<sub>2</sub>O, from 8 cmH<sub>2</sub>O (5.88 mmHg) to 32 cmH<sub>2</sub>O (23.54 mmHg),

by increasing the height of a column of fluid of BSS. A steady-state was achieved after 10 min. Stabilization between all subsequent steps was obtained within 5 min. Flow was constantly measured via a microfluidic flow sensor (0.07–1.5  $\mu$ l/min, MFS1; Elveflow, Paris, France). For analysis, flow rates at each pressure level were plotted, and the slope of the regression line was used to calculate the outflow facility for each eye. Any eyes that developed visible leaks during outflow facility determination, or that had pressure-flow correlations with  $R^2 < 0.9$ , were excluded from analysis but were used for RNA quantification.

**Tissue collection and confirmation of transgene expression:** After outflow facility determination in adenovirus-treated mice, mCherry expression in the TM was determined in all AdPLAT- and AdPLATNE-injected eyes. The eyes were dissected on ice to isolate a rim of tissue containing the TM by removing most of the iris and ciliary body. Fluoroshield with diamidino-2-phenylindole (DAPI) histology mounting medium (Millipore Sigma, St. Louis, MO) was applied for the counterstain. Flat mounts of the rims containing TM were observed in an epifluorescent microscope equipped with the appropriate filter sets to visualize mCherry and DAPI expression. After observation, dissected rims were immediately immersed in an RNA stabilizing agent (RNAlater, Invitrogen by ThermoFisher Scientific) and frozen. For TM tissue collection following protein treatment, all eyes were flash frozen in liquid nitrogen and subsequently dissected on ice to obtain the angle ring containing the TM tissues, as previously described [43]. Dissected TM tissues were immersed in RNAlater solution, snap-frozen, and stored at  $-80^\circ\text{C}$  until RNA extraction.

**RNA isolation and quantitative real-time PCR:** The collected tissue was pooled (four eyes) and homogenized in TRIzol reagent (Life Technologies, Carlsbad, CA). RNA was isolated per the manufacturer's instructions and resuspended in nuclease-free water. RNA concentration was determined with

a Nanodrop ND-1000 Spectrophotometer (ThermoFisher Scientific). cDNA was synthesized using a High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Waltham, MA) according to the manufacturer's protocol. Quantitative real-time PCR (qRT-PCR) was performed using Green-2-Go qPCR Mastermix-ROX (BioBasic, Amherst, NY) on a QuantStudio 6 Flex thermal cycler (Applied Biosystems, Carlsbad, CA).

The mRNA expression of sheep *PLAT* and mouse *Mmp-2*, *Mmp-9*, and *Mmp-13* in angle ring tissues was determined. Primers were designed using Primer-BLAST (in the public domain), and their specificity was confirmed by the presence of a single band of the expected size on agarose gel electrophoresis. Primer sequences are listed in Table 1. Specificity was further verified during each experiment by inspection of melting curves to ensure the absence of multiple-sized amplification products. The annealing temperature was  $60^\circ\text{C}$ .

**Statistical analysis:** The values of target mRNA expression were normalized to the expression levels of *18S* (18S rRNA). The relative fold change was calculated by using the  $\Delta\Delta\text{Ct}$  method [49]. The presence of outliers was tested using the Thompson Tau test, and any outliers were removed from the analysis. The results were subjected to one-way ANOVA for treatment using number cruncher statistical systems (NCSS) statistical software. For outflow facility and IOP experiments, a *t*-test or one-way ANOVA was used where appropriate. Significant differences ( $p < 0.05$ ) were explored with Tukey-Kramer post hoc analysis.

## RESULTS

**Confirmation of lack of enzymatic activity of cells transfected with *PLAT* (1677T>G):** To confirm that the mutant *PLAT* (1677T>G) does not generate an enzymatically active tPA protein, cell culture supernatant from transfected HMVECs

TABLE 1. PRIMER SEQUENCES OF GENES ANALYZED BY qRT-PCR.

Number	Gene	Sequence (5'-3')
1	<i>PLAT</i>	FP: CAGTGCCCAGAAGGGTTCAT RP: GTAGCACCAGGGCTTTGAGT
2	<i>Mmp-2</i>	FP: ACAGTGACACCACGTGACAA RP: GGTCAGTGGCTTGGGGTATC
3	<i>Mmp-9</i>	FP: GCGTCGTGATCCCCACTTAC RP: CAGGCCGAATAGGAGCGTC
4	<i>Mmp-13</i>	FP: TACCATCCTGCGACTCTTGC RP: TTCACCCACATCAGGCACTC
5	<i>18S</i>	FP: AGTCCCTGCCCTTTGTACACA RP: GATCCGAGGGCCCTCACTAAAC

FP, forward primer; RP, reverse primer



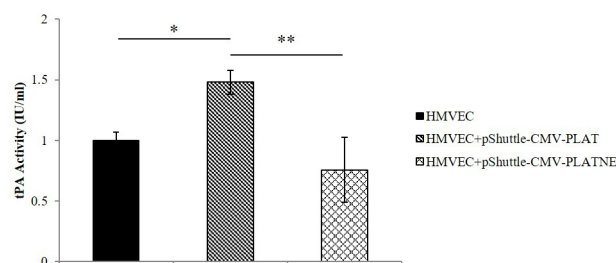


Figure 2. Quantification of tPA enzymatic activity. tPA enzymatic activity in supernatants of non-transfected HMVECs (n = 3) and HMVECs transfected with either pShuttle-CMV-PLAT (n = 3) or pShuttle-CMV-PLATNE (n = 4). tPA enzymatic activity in supernatant from pShuttle-CMV-PLAT

transfected cells was significantly higher than the activity in supernatant from pShuttle-CMV-PLATNE transfected and non-transfected cells ( $p < 0.01$ , ANOVA). Asterisks indicate differences on Tukey-Kramer post hoc analysis, \*  $p < 0.05$ , \*\*  $p < 0.01$ .

was assayed for its ability to catalyze the conversion of plasminogen to plasmin. The supernatant of cells transfected with pShuttle-CMV-PLAT had significantly higher enzymatic activity ( $p < 0.01$ , ANOVA) when compared to the supernatant from pShuttle-CMV-PLATNE and that from non-transfected cells ( $p < 0.01$  and  $p < 0.05$ , respectively; Tukey-Kramer post hoc analysis), while the enzymatic activity of the supernatant from pShuttle-CMV-PLATNE transfected cells was not different from that of non-transfected cells ( $p > 0.05$ , Tukey-Kramer post hoc analysis; Figure 2).

#### PLAT (and PLATNE) expression in adenovirus-injected eyes:

In animals injected with adenoviral vectors, mCherry expression was distributed in many of the cells along the entire

length of the TM in AdPLAT- (Figure 3A) and AdPLATNE- (Figure 3B) treated eyes. Expression of the *PLAT* gene was detected by qRT-PCR in the TM of eyes receiving AdPLAT and AdPLATNE, respectively, while *PLAT* expression was below the detection limits in naïve and AdNull treated eyes (Figure 3C).

*AdPLAT and AdPLATNE attenuate steroid-induced outflow facility reduction and increase Mmp expression:* In eyes treated with TA and adenovirus, the mean  $\pm$  standard deviation outflow facility ( $\mu\text{l}/\text{min}/\text{mmHg}$ ) was  $100.9 \pm 18.7 \times 10^{-5}$  in AdPLAT (n = 11),  $101.3 \pm 28 \times 10^{-5}$  in AdPLATNE (n = 8), and  $63.6 \pm 33.8 \times 10^{-5}$  in AdNull (n = 11) treated eyes, while the outflow facility was  $117 \pm 28.3 \times 10^{-5}$  in naïve C57BL/6J

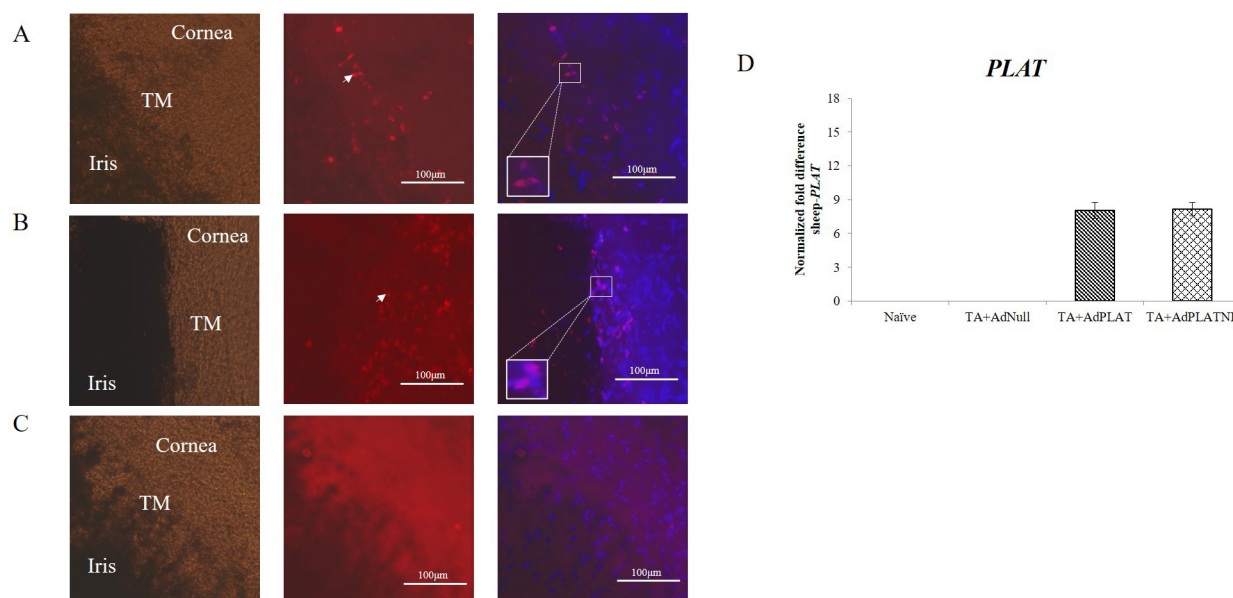


Figure 3. Visualization of adenovirus expression. Flatmounts of anterior segments of mouse eyes from animals injected with (A) AdPLAT (TA+AdPLAT), (B) AdPLATNE (TA+AdPLATNE), and (C) AdNull (TA+AdNull). There was robust mCherry expression in both AdPLAT- and AdPLATNE-treated eyes (white arrowheads). There was no mCherry expression in AdNull-treated eyes. DAPI was used as a counterstain for nuclei. Arrows indicate mCherry-positive cells. C: qRT-PCR quantification of *PLAT* expression in angle ring tissues. Levels of *PLAT* were detected in AdPLAT (n = 16) and AdPLATNE (n = 12) eyes, but were undetectable in AdNull (n = 16) and naïve (n = 20) eyes. TM = trabecular meshwork.

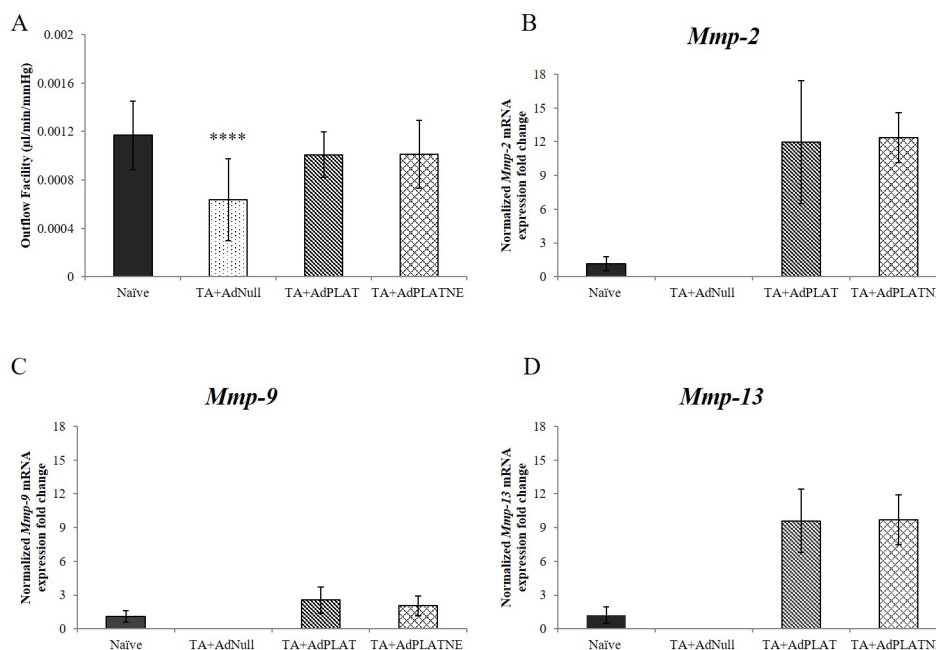


Figure 4. Outflow facility was significantly enhanced in adenovirus-treated C57BL/6 mouse eyes. **A:** Outflow facility in TA- and adenovirus-treated C57BL/6J mouse eyes. Outflow facility (mean  $\pm$  SD  $\mu$ l/min/mmHg) was significantly increased in eyes treated with TA+AdPLAT (n = 11) and TA+AdPLATNE (n = 8) compared to those treated with TA+AdNull (n = 11; \*\*\*\* p<0.0001, ANOVA with Tukey-Kramer post hoc analysis). The outflow facility of naïve (not treated with either TA or adenovirus) C57BL/6 animals is included for comparison. The outflow facility in these eyes was similar to the outflow facility in AdPLAT- and AdPLATNE-treated eyes. Gene expression changes in *Mmp-2* (**B**), *Mmp-9* (**C**), and *Mmp-13* (**D**) were normalized (mean  $\pm$  SD) to values from naïve eyes. Expression was significantly different between TA+AdNull eyes (n = 16) and both TA+AdPLAT eyes (n = 16) and TA+AdPLATNE eyes (n = 8). Naïve eyes (n = 20) also had lower expression than the TA+AdPLAT- and TA+AdPLATNE-treated eyes. Asterisks indicate differences on Tukey-Kramer post hoc analysis, \*\* p<0.01, \*\*\*p<0.001, \*\*\*\*p<0.0001. mRNA was below detectable limits for *Mmp-2*, *Mmp-9*, and *Mmp-13* in the TA+AdNull group.

eyes (n = 10; p<0.0001, ANOVA). Similar to previous reports [47], TA caused a ~46% reduction in outflow facility from baseline, while treatment with AdPLAT or AdPLATNE restored outflow facility to baseline levels in TA-exposed eyes. AdNull-treated eyes had a significantly lower outflow facility than all other groups (p<0.0001, Tukey-Kramer post hoc analysis), while there was no significant difference between naïve, TA+AdPLAT, or TA+AdPLATNE groups (p>0.05, Tukey-Kramer post hoc analysis; Figure 4A).

The expression of *Mmp-2*, *Mmp-9*, and *Mmp-13* in C57BL/6J eyes exposed to TA and AdNull (n = 16) was below detection levels. Treatment with AdPLAT (n = 16) and AdPLATNE (n = 8) caused an upregulation in *Mmp-2* (Figure 4B), *Mmp-9* (Figure 4C), and *Mmp-13* (Figure 4D) expression (p<0.0001, p<0.001, and p<0.0001, respectively; ANOVA with Tukey-Kramer post hoc analysis). *Mmp* expression after AdPLAT and AdPLATNE appeared to be higher than that in naïve eyes (n = 20).

*Both enzymatically active and enzymatically inactive tissue plasminogen activators attenuate steroid-induced outflow*

*facility reduction and increase Mmp expression:* In eyes treated with TA and the respective protein injection, the mean  $\pm$  standard deviation outflow facility ( $\mu$ l/min/mmHg) was  $67.8 \pm 25.8 \times 10^{-5}$  in TA+BSA (n = 18),  $105.3 \pm 35.8 \times 10^{-5}$  in TA+tPA (n = 15), and  $114 \pm 40.3 \times 10^{-5}$  in TA+NE-tPA (n = 10; p<0.0001, ANOVA). Treatment with tPA or NE-tPA restored outflow facility to naïve baseline levels. There was a significantly lower outflow facility in TA+BSA eyes compared to that in all other groups (p<0.0001, Tukey-Kramer post hoc analysis). Furthermore, there was no significant difference between naïve, TA+tPA, and TA+NE-tPA groups (p>0.05, Tukey-Kramer post hoc analysis; Figure 5A).

tPA- (n = 8) and NE-tPA- (n = 8) treated eyes showed a significant upregulation in *Mmp-2* (Figure 5B), *Mmp-9* (Figure 5C), and *Mmp-13* (Figure 5D) expression (p<0.0001, p<0.01, and p<0.01, respectively; ANOVA with Tukey-Kramer post hoc analysis) compared to BSA- (n = 4) treated controls and appeared to be higher than that in naïve eyes (n = 20).

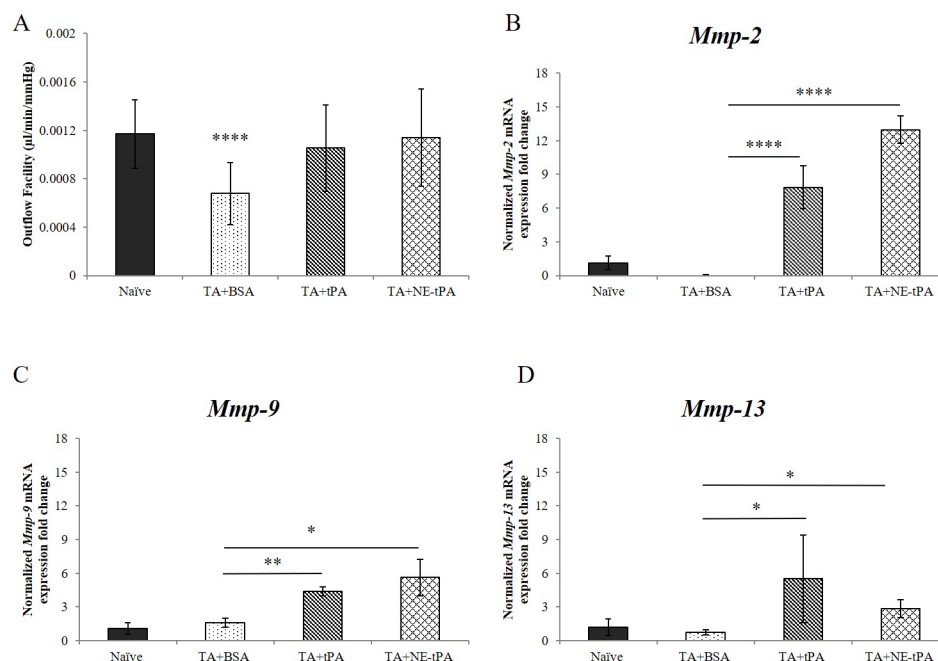


Figure 5. Outflow facility and *Mmp* gene expression were significantly enhanced in protein-treated C57BL/6 mouse eyes. **A:** Outflow facility in TA- and protein-treated C57BL/6 mouse eyes. Outflow facility (mean  $\pm$  SD  $\mu$ l/min/mmHg) was significantly increased in eyes treated with TA+tPA (n = 15) and TA+NE-tPA (n = 10) compared to those treated with TA+BSA (n = 18; \*\*\*\* p<0.0001, ANOVA with Tukey-Kramer post hoc analysis). The outflow facility of naïve (not treated with either TA or protein) C57BL/6J animals is included for comparison. The outflow facility in these eyes was similar to the outflow facility in tPA- and NE-tPA-treated eyes. Gene expression changes in *Mmp-2* (**B**), *Mmp-9* (**C**), and *Mmp-13* (**D**) were normalized (mean  $\pm$  SD) to values from naïve eyes. Group means were significantly different in TA+BSA eyes (n = 4), TA+tPA eyes (n = 8), and TA+NE-tPA eyes (n = 8; ANOVA, *Mmp-2*, p<0.0001, *Mmp-9*, p<0.0001, *Mmp-13*, p<0.01). Asterisks indicate differences in Tukey-Kramer post hoc analysis, \* p<0.05, \*\*p<0.01, \*\*\*p<0.001 \*\*\*\*p<0.0001.

*Both enzymatically active and enzymatically inactive tissue plasminogen activator administration rescues outflow facility in PlatKO mice with an upregulation in Mmp-9 and Mmp-13 expression:* In *PlatKO* mice receiving intravitreal BSA injections (n = 19), the mean  $\pm$  standard deviation outflow facility ( $\mu$ l/min/mmHg) was  $61 \pm 22 \times 10^{-5}$ . Intravitreal administration of tPA or NE-tPA caused a 46% ( $89 \pm 27 \times 10^{-5}$ ) and 51% ( $92 \pm 42 \times 10^{-5}$ ) increase in outflow facility, respectively, compared to the outflow facility of BSA-treated eyes. There was a significant difference in outflow facility among *PlatKO*+BSA, *PlatKO*+tPA, and *PlatKO*+NE-tPA eyes (p<0.001, ANOVA), with outflow facility in *PlatKO*+BSA eyes significantly lower in comparison to the two other groups (p<0.01, Tukey-Kramer post hoc analysis; Figure 6A).

Intravitreal tPA and NE-tPA administration in *PlatKO* mice caused a significant upregulation in *Mmp-9* (Figure 6C) expression (p<0.0001, ANOVA) compared to that in eyes treated with BSA (n = 8; p<0.01, p<0.0001, Tukey-Kramer post hoc analysis). There was also a significant upregulation in *Mmp-13* (Figure 6D) expression (p<0.0001, ANOVA) in tPA- and NE-tPA-treated *PlatKO* eyes compared to

BSA-treated eyes (p<0.0001, p<0.05, Tukey-Kramer post hoc analysis). *Mmp-2* (Figure 6B) expression was not significantly different in tPA- and NE-tPA-treated *PlatKO* eyes compared to expression in BSA-treated eyes (p>0.05, ANOVA).

*Tissue plasminogen activator does not alter baseline outflow facility, Mmp, or Plat expression:* Treatment with intravitreal tPA and NE-tPA did not cause further enhancement in outflow facility in C57BL/6J mouse eyes not previously exposed to steroids (p>0.05, ANOVA). The mean  $\pm$  standard deviation outflow facility ( $\mu$ l/min/mmHg) was  $101.6 \pm 27.1 \times 10^{-5}$ ,  $102.7 \pm 19.5 \times 10^{-5}$ , and  $110 \pm 36.5 \times 10^{-5}$  in BSA (n = 19), tPA (n = 11), and NE-tPA (n = 11) treated eyes, respectively (Figure 7A). No significant changes in *Mmp* gene expression were detected between treatment groups (p>0.05, ANOVA; Figure 7B–D). Furthermore, exogenous tPA and NE-tPA did not cause significant changes in endogenous *Plat* expression (p>0.05, ANOVA; Figure 7E).

*Tissue plasminogen activator does not rescue outflow facility reduction in Mmp-9KO mice:* Similar to previous reports [50], intraocular pressure was significantly elevated (~50%) in *Mmp-9KO* mice ( $21.28 \pm 2.07$  mmHg; n = 20) when compared

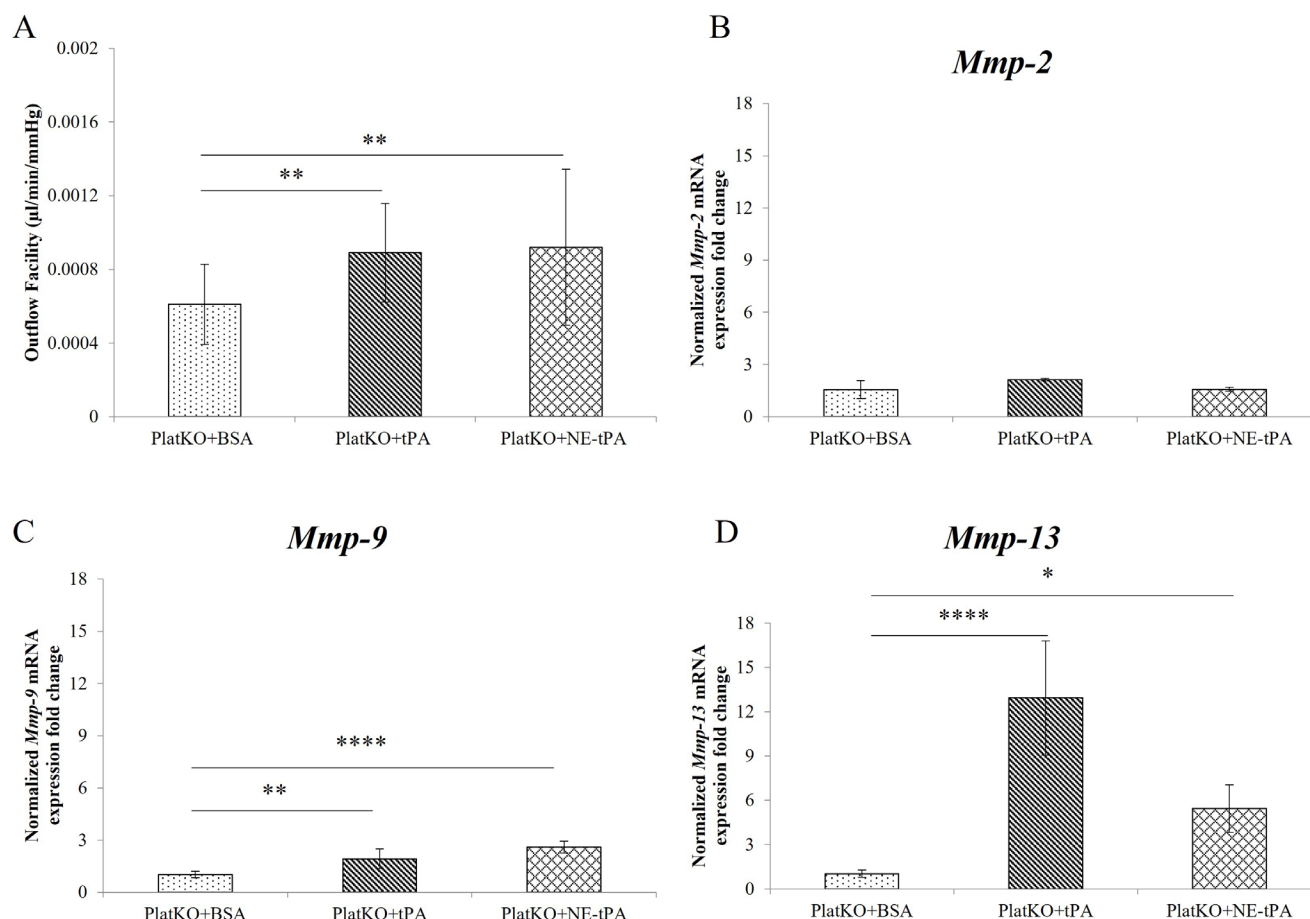


Figure 6. Outflow facility and *Mmp* gene expression were significantly enhanced in protein-treated *PlatKO* mouse eyes. **A:** Outflow facility in protein-treated *PlatKO* mouse eyes. Outflow facility (mean  $\pm$  SD  $\mu$ l/min/mmHg) was significantly increased in eyes treated with tPA (n = 12) and NE-tPA (n = 13) compared to those treated with BSA (n = 19; \*\* p<0.01, ANOVA with Tukey-Kramer post hoc analysis). Group means of gene expression changes in *Mmp-9* (**C**) and *Mmp-13* (**D**) were significantly different in *PlatKO* BSA eyes (n = 8), tPA eyes (n = 12), and NE-tPA eyes (n = 8; ANOVA, *Mmp-9*, p<0.0001, *Mmp-13*, p<0.0001). Group means of gene expression changes in *Mmp-2* (**B**) were not significantly different across treatment groups (ANOVA, *Mmp-2*, p>0.05). Asterisks indicate differences in Tukey-Kramer post hoc analysis, \* p<0.05, \*\* p<0.01, \*\*\* p<0.001, \*\*\*\* p<0.0001.

to C57BL/6J mice ( $14.14 \pm 0.44$  mmHg; n = 20; Figure 8A). Furthermore, the mean  $\pm$  standard deviation outflow facility ( $\mu$ l/min/mmHg) was significantly reduced in *Mmp-9*KO mice ( $51.4 \pm 19.9 \times 10^{-5}$ ; n = 14) compared to C57BL/6J mice ( $124.4 \pm 29.2 \times 10^{-5}$ ; n = 16; p<0.0001, *t* test; Figure 8B). Treatment with intravitreal tPA or NE-tPA did not increase outflow facility in *Mmp-9*KO mouse eyes (p>0.05, ANOVA; Figure 8C).

## DISCUSSION

Steroid-induced IOP elevation can occur with topical corticosteroids in approximately 30% of the population [10]. Prior work to understand the pathophysiology of this condition has implicated the ECM in outflow pathways [7,11,14,15,25,51].

Plaque material accumulation has been detected in TM specimens of patients with steroid-induced IOP elevation [17], as well as in steroid-treated cultured TM cells [12,14]. Similar material has also been detected in animal models of the disease, such as cows and mice [13,16]. This accumulation of ECM material suggests that, at least for some individuals, treatment with steroids leads to either excess ECM deposition or dysregulation of normal ECM turnover.

We previously reported that the expression of tPA (one of the enzymes regulating ECM turnover) is reduced following prednisolone exposure in sheep outflow tissues [20]. In addition, intraocular tPA administration can reverse steroid-induced outflow facility reduction in mice and IOP elevation in sheep [20,43], confirming that steroid-induced tPA changes



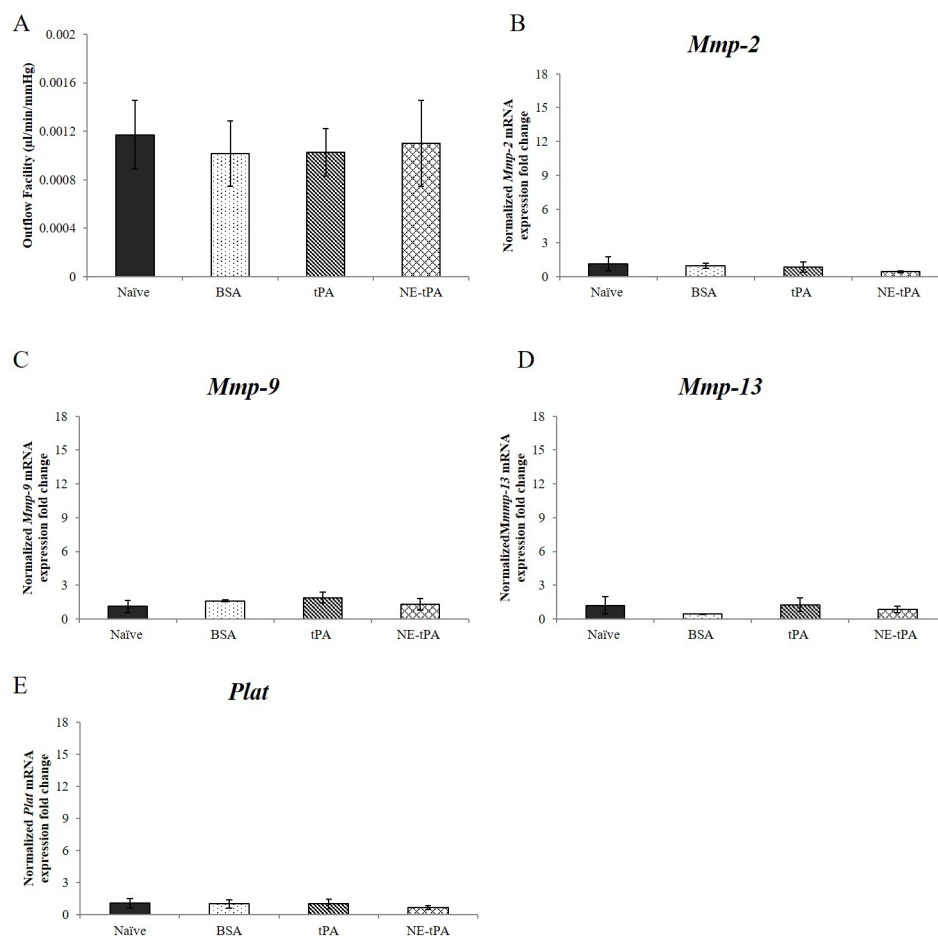


Figure 7. Baseline outflow facility and *Mmp* gene expression were not altered in C57BL/6 mouse eyes. **A:** Outflow facility in protein-treated C57BL/6 mouse eyes. Outflow facility (mean  $\pm$  SD  $\mu$ l/min/mmHg) was unchanged following treatment in non-steroid-treated eyes exposed to tPA (n = 11) and NE-tPA (n = 11) compared to those treated with BSA (n = 19;  $p > 0.05$ , ANOVA). The outflow facility of naïve (not treated with protein) C57BL/6 animals is included for comparison. The outflow facility in these eyes was similar to the outflow facility in tPA-, NE-tPA-, or BSA-treated C57BL/6 eyes. Group means of gene expression changes in *Mmp-2* (**B**), *Mmp-9* (**C**), *Mmp-13* (**D**), and *Plat* (**E**) were not significantly different in BSA eyes (n = 12), tPA eyes (n = 8), or NE-tPA eyes (n = 8;  $p > 0.05$ , ANOVA).

may be responsible for some of the ECM accumulation seen in this condition. Steroid-induced tPA expression reduction is paralleled by a decrease in the expression of MMPs [52]. Furthermore, the exogenous supplementation of tPA seems to reverse changes in MMP expression [43], making it tempting to speculate that the effects of tPA on outflow facility are mediated via its actions on MMP expression.

tPA is a serine protease that is better known for its action in regulating the fibrinolytic pathway. It lies upstream and can activate plasminogen into plasmin, which then degrades fibrin to dissolve blood clots [26]. Yet, tPA has other roles in tissue homeostasis, either through plasmin or through independent activity. tPA has, in addition to its enzymatic activity (which can directly or indirectly affect ECM components), non-enzymatic domains within its protein structure

[33-35] that can bind to distinct receptors, eliciting a specific cellular response. Since we have previously reported that plasminogen levels were below detection limits in anterior segment outflow tissues in mice [44], this work is an effort to determine whether direct enzymatic action by tPA is required for its effect on outflow facility, or whether these effects are solely the result of cytokine events. To obtain an answer, we used enzymatically inactive tPA.

The enzymatic activity of tPA has been well characterized and is dependent on the presence of an active site serine in position 478 [38]. Conversion of the active site serine-478 to an alanine completely abolishes the protein's enzymatic activity [39,40], but still allows tPA to bind to receptors (i.e., LRP-1), inhibitors (i.e., PAI-1), and ligands (i.e., plasminogen) [41].

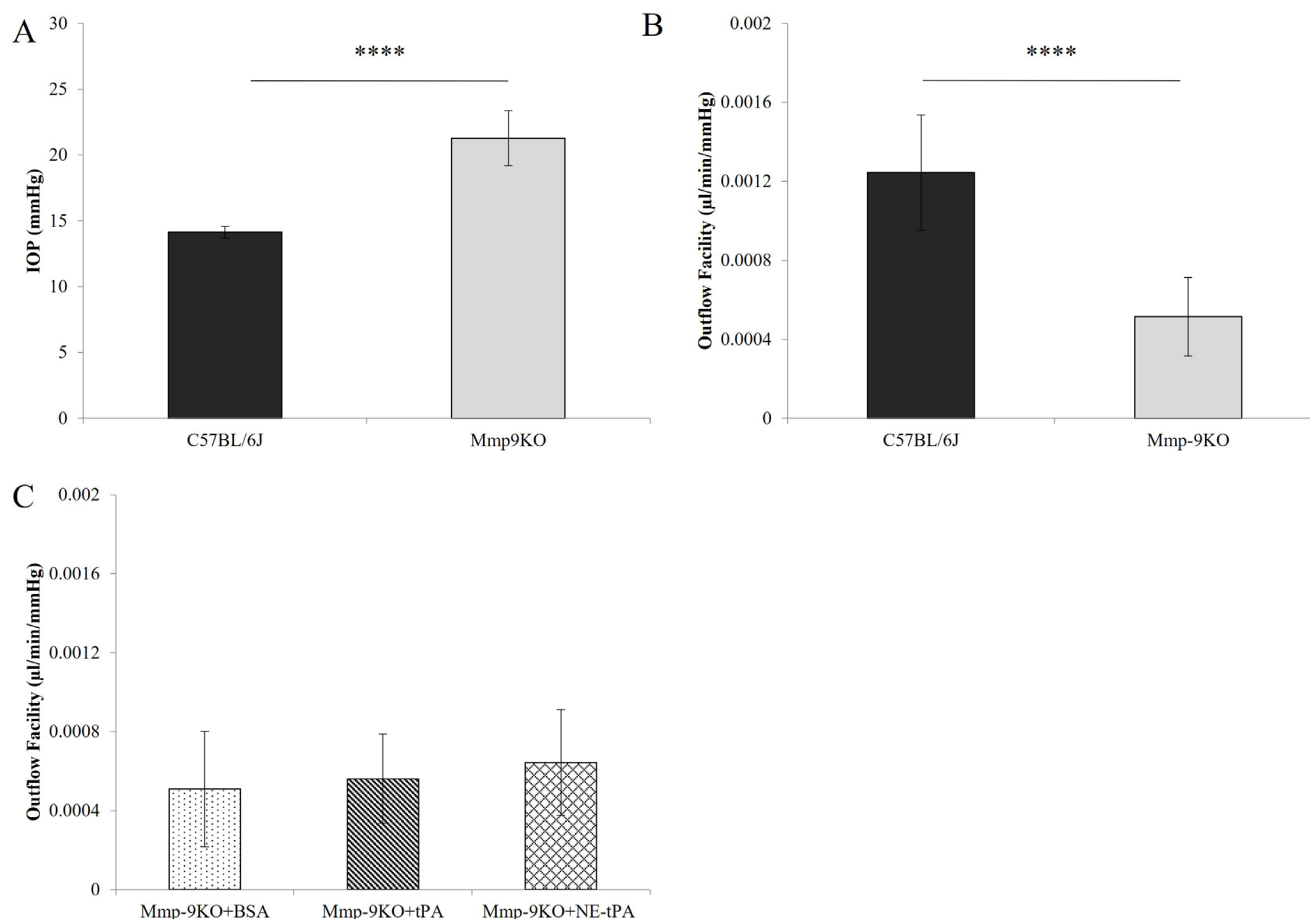


Figure 8. Protein treatment did not affect outflow facility in *Mmp-9KO* mouse eyes. **A:** Intraocular pressure in *Mmp-9KO* and C57BL/6 mouse eyes. Intraocular pressure (mean  $\pm$  SD mmHg) in *Mmp-9KO* mouse eyes ( $n = 20$ ) was significantly higher than that of C57BL/6J animals ( $n = 20$ ; \*\*\*\*  $p < 0.0001$ ,  $t$  test). **B:** Outflow facility in *Mmp-9KO* and C57BL/6 mouse eyes. Outflow facility (mean  $\pm$  SD  $\mu\text{l}/\text{min}/\text{mmHg}$ ) in *Mmp-9KO* mouse eyes ( $n = 14$ ) was significantly lower than that of C57BL/6J animals ( $n = 16$ ; \*\*\*\*  $p < 0.0001$ ,  $t$  test). **C:** Outflow facility in BSA-, tPA-, and NE-tPA-treated *Mmp-9KO* mouse eyes. Outflow facility (mean  $\pm$  SD  $\mu\text{l}/\text{min}/\text{mmHg}$ ) was not significantly different across groups: *Mmp-9KO*+BSA ( $n = 21$ ), *Mmp-9KO*+tPA ( $n = 13$ ), and *Mmp-9KO*+NE-tPA ( $n = 8$ ;  $p > 0.05$ , ANOVA).

To complement our existing molecular tools, we first created a clone of the enzymatically inactive version of sheep *PLAT* by site-directed mutagenesis. To confirm that it encoded for a protein lacking enzymatic activity, we transfected cultured HMVECs and assayed, in the supernatant, the ability of tPA to convert plasminogen into plasmin. Transfection with the non-mutant sheep *PLAT* significantly increased tPA activity in the HMVEC supernatant in comparison to transfection with the enzymatically inactive mutant sheep *PLAT*. Given the relatively low transfection efficiency of such large plasmids, such differences were highly significant.

Since transfection of outflow pathways with plasmids is challenging (if not impossible), we used adenoviral transfection to achieve transgene expression in these tissues. Adenoviral transfection is effective in achieving at least short-term

(1–2 weeks) expression of transgenes in the TM and has been used by us and others for that reason [43,53,54]. Although adenoviral injections in the anterior chamber can elicit a significant inflammatory response, concurrent use of steroids alleviates this effect and allows robust transfection and transgene expression. The use of an adenoviral vector carrying a fluorescent protein (mCherry), as well as the molecular quantification of the transgenic transcript by qRT-PCR, allowed us to confirm the successful transfection of TM cells in vivo.

Using this approach, we confirmed our previously published results [43] that *PLAT* overexpression in the TM abrogates steroid-induced outflow facility reduction in mice. Surprisingly, the mutant *PLAT* that encoded for a protein without enzymatic activity, as shown by the chromogenic

activity assay, was equally effective in restoring outflow facility in steroid-treated mouse eyes.

To further confirm that the observed effect of using adenoviral transfection is mediated via the tPA protein, we also administered (in another set of animals) either enzymatically active or enzymatically inactive tPA. As with the virally transfected eyes, both enzymatically active and enzymatically inactive tPA completely restored the outflow facility that was reduced by steroid treatment.

Importantly, enzymatically active and enzymatically inactive tPA (either by transfection or by direct protein supplementation) caused similar upregulation of *Mmp-2*, *Mmp-9*, and *Mmp-13* expression in steroid-treated eyes. Interestingly, these *Mmps* were upregulated to levels even higher than those detected in naïve (not exposed to steroid) mouse eyes. These findings indicate that the classic fibrinolytic serine protease activity of tPA [26] is not critical for its effects on aqueous humor outflow regulation. Given that plasminogen expression has been shown to be below detectable limits in angle ring tissues containing the TM [44], it is unlikely that tPA regulates outflow facility via enzymatic activity, suggesting that tPA functions as a cytokine to induce changes in *Mmp* transcription [32-34], which ultimately results in the modulation of outflow. Though not previously described in TM tissue, this tPA-mediated transcriptional regulation of MMPs has been shown in other tissues [32-34,37].

To further confirm that tPA acts via a non-enzymatic mechanism to affect outflow facility, we used *PlatKO* mice. These mice have undetectable tPA enzymatic activity and display a significant reduction in outflow facility [44] compared with wild-type littermates. As expected, intravitreal administration of tPA in these animals increased outflow facility to levels similar to those of C57BL/6J mice (the background strain on which *PlatKO* mice were maintained). Importantly, enzymatically inactive tPA was equally effective in improving the outflow facility in these animals. Administration of enzymatically active and inactive tPA significantly increased *Mmp-9* levels in the outflow tissues of these animals. In addition, enzymatically active tPA caused an upregulation (to a different degree) in the expression of *Mmp-13*. Such changes have also been reported for tPA in other tissues [55-57].

The relationship between tPA and MMP-9 has been studied extensively in the context of its therapeutic dosing for acute thrombotic cerebrovascular events. tPA administration results in enhanced MMP-9 activation, which can lead to significant adverse outcomes [56-58]. However, in the context of aqueous flow, upregulation or activation of *Mmp-9* may have beneficial effects. To determine whether tPA-induced

*Mmp-9* expression upregulation is critical for mediating its effects on outflow facility regulation, we used *Mmp-9*-deficient (*Mmp-9KO*) mice. These mice were originally described in studies of carcinogenesis [46] and are viable and fertile. Although they have diminished neuroretinal degeneration [59], they show no obvious (clinical) ocular phenotype. They have significantly higher IOP than their wild-type littermates [50,60], and we have confirmed this IOP elevation in comparison to C57BL/6J mice. Furthermore, this IOP elevation is caused by a significant reduction in outflow facility, corroborating previous findings on aqueous turnover in these mice [60]. Intravitreal administration of either enzymatically active or enzymatically inactive tPA failed to increase outflow facility in these animals, suggesting that *Mmp-9* functions downstream of tPA to affect aqueous outflow, and confirming a role of *Mmp-9* in the regulation of outflow facility.

Given the detrimental effects of tPA-induced MMP-9 upregulation in the brain [56-58], we also explored whether tPA increases *Mmp-9* expression under baseline conditions (in the absence of steroids), potentially resulting in an excessive increase in outflow facility that could lead to hypotony. Surprisingly, administration of either enzymatically active or enzymatically inactive tPA in C57BL/6J mice did not affect outflow facility or *Mmp-9* expression, suggesting that its actions may be regulated by availability of its receptor or other downstream regulatory molecules [61].

The findings of this study provide a potential mechanism by which tPA is able to regulate the outflow of aqueous humor following exposure to steroids. tPA likely functions as a cytokine to bind to a cell surface receptor and alter downstream intracellular signaling, as evidenced by the equal efficacies of enzymatically active and enzymatically inactive tPA in reversing steroid-induced outflow facility reduction in C57BL/6J mice and *PlatKO* mice. These physiologic effects are correlated with the enhanced expression of *Mmp-9*, which likely occurs via downstream transcriptional regulation, as evidenced by the inability to enhance outflow facility in *Mmp-9KO* mice. The potential relationship between *Mmp-2* and *Mmp-13* expression and outflow facility regulation remains unclear. Previous studies with other tissues have shown that MMP-13 may participate in the proteolytic activation of pro-MMP-9 [62-64]. If this is indeed the mechanism of action of *Mmp-13* in the mouse outflow system, it would explain why outflow facility is reduced in *Mmp-9KO* animals, even if *Mmp-13* expression may become upregulated as a result of tPA or NE-tPA treatment. The role of MMP-2 (gelatinase A) in glaucoma has been unclear. There is evidence indicating that the MMP-2 present in the

aqueous humor remains inactive in its pro-peptide form [65] and as such may not directly affect the outflow facility process. Future experiments to discern the contribution of MMP activity to outflow facility regulation could involve the use of *Mmp*KO mice or MMP pharmacologic inhibitors.

LRP-1 is a potential receptor candidate linking extracellular tPA and *Mmp* transcription, as has been reported in other organ systems [33-35] and is expressed in TM cells [66]. LRP-1 is a scavenger receptor most classically linked to receptor-mediated endocytosis in lipoprotein metabolism [34]. Beyond this role, however, LRP-1 is associated with downstream intracellular signaling cascades and has a high affinity for binding tPA [33-35,67]. Both enzymatically active and enzymatically inactive tPA have been shown to bind to cell surface LRP-1 in complex with NMDA-R [36,68] to initiate its phosphorylation and subsequent extracellular-signal related activation of kinases 1/2 (Extracellular-signal related activation of kinases 1/2 [ERK1/2]), leading to downstream gene expression changes [33,68], including MMP-9 [33,34].

In addition, LRP-1 has been reported to mediate the endocytosis and degradation of excess tPA [69]. Such an action may explain the fact that exogenously applied tPA (either enzymatically active or enzymatically inactive) failed to increase outflow facility or alter *Mmp* expression under baseline conditions in C57BL/6J mice. Finally, it is worth noting that another member of the LRP gene family (LRP-2), which is also a potential receptor for tPA, has been implicated in glaucoma pathophysiology, as it has been reported that *lrp2* deletion causes significant IOP elevation in zebrafish [70]. Identifying potential tPA receptors in TM tissue will require additional work.

In summary, we have shown that the enzymatic activity of tPA is not essential for its action on regulating aqueous outflow and that its action is, at least in part, mediated by transcriptional control of *Mmp-9*. Previous studies on the role of intraocular MMP-9 indicate that its expression is necessary for proper collagen turnover at the iridocorneal angle [60]. Although tPA-dependent *Mmp* expression upregulation does not prove subsequent enzymatic activity at the TM, it suggests such a mechanism of action. Furthermore, tPA (either enzymatically active or inactive) does not affect outflow facility in mice under baseline conditions, making it an appealing target for therapeutic development of treatment for elevated IOP in steroid-induced glaucoma.

## ACKNOWLEDGMENTS

**Funding:** This work was supported by the National Institutes of Health [grant number R01EY025543]. Funding agencies had no involvement in study design; in the collection, analysis and interpretation of data; in the writing of the report; and in the decision to submit the article for publication. **Author contributions statement:** S.G. and J.D. designed research; S.G., A.O.B., Y.H. and A.D. performed research; S.G. analyzed data; and S.G. wrote the paper; all authors reviewed the manuscript.

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Articles are provided courtesy of Emory University and the Zhongshan Ophthalmic Center, Sun Yat-sen University, P.R. China. The print version of this article was created on 7 December 2021. This reflects all typographical corrections and errata to the article through that date. Details of any changes may be found in the online version of the article.