BRIEF COMMUNICATION

Inhibition of CD40-TRAF6-dependent inflammatory activity halts the onset of diabetic retinopathy in streptozotocin-diabetic mice

Scott J. Howell1,2, Chieh A. Lee1, Thomas E. Zapadka1,2, Sarah I. Lindstrom1, Brooklyn E. Taylor1, Zakary R. R. Taylor1, Katherine G. Barber2 and Patricia R. Taylor1,2,3✉

This is a U.S. Government work and not under copyright protection in the US; foreign copyright protection may apply 2022

INTRODUCTION

Inflammation can impair the retinal microvasculature, and lead to diabetic retinopathy [1]. IL-17A is an inflammatory protein that plays a role in the onset of diabetic retinopathy [2]. Normally, IL-17A is not constitutive [3]. However, it is continuously produced in diabetics [4]. Recently, we reported that IL-17A enhances retinal vascular leakage and capillary degeneration in diabetic mice [2, 5]. When IL-17A binds to its receptor, it can initiate TNF Receptor Associated Factor 6 (TRAF6) to bind to CD40. The CD40-TRAF6 complex then initiates NFκB activation, inflammation, and tissue damage [6]. Both CD40 and TRAF6 are constitutively expressed in the retina, and upregulated during diabetes [7, 8]. Thus, we hypothesized that the CD40-TRAF6 axis could be an optimal therapeutic target for early-stage diabetic retinopathy.

We investigated the CD40-TRAF6 axis in the onset of diabetic retinopathy in streptozotocin (STZ)-induced diabetic mice using a small molecule inhibitor (SMI-6877002), which can pass the blood-retina barrier [9, 10]. SMI-6877002 causes a conformational change in the binding groove of TRAF6 upon CD40 binding, which inhibits the CD40-TRAF6 complex from initiating NFκB activation [9]. This causes a decrease in inflammation and suppresses TNF-α, which is produced in early-stage retinal pathogenesis and a precursor to diabetic retinopathy [9–11]. A weekly subcutaneous injection of 100 μl of saline containing 20 μM of SMI-6877002 was sufficient to halt TNF-α and VEGF production in Muller glia and the retina. When SMI-6877002 was administered throughout an 8-month duration in STZ-diabetic mice, retinal vascular leakage and capillary degeneration was ameliorated in diabetic mice. Collectively, these data suggest that the CD40-TRAF6 axis plays a pivotal role in the onset of diabetic retinopathy, and could be a novel therapeutic target for early diabetic retinopathy.

MATERIALS & METHODS

Diabetic mice

Streptozotocin (60 mg/kg) was injected on five consecutive days in 8–10 week-old C57BL/6 mice as previously described [12, 13]. Diabetes was confirmed by blood glucose higher than 250 mg/dl and hemoglobin A1C levels. SMI-6877002 treatment was administered after streptozotocin damaged the pancreatic beta cells, which allows diabetic retinopathy to develop. CWRU IACUC approved animal protocols with a power calculation = 0.9, which have been strictly followed.

SMI-6877002 treatment

SMI-6877002 (3-((2,5-Dimethylphenyl) amino)-1-phenyl-2-propen-1-one, (2E)-3-((2,5-Dimethylphenyl) amino)-1-phenyl-2-propen-1-one) is a cell-permeable propenone that causes a conformational change in the Arg466 residue, altering the binding groove of the
CD40-TRAF6 complex. This halts NFκB activation and reduces inflammation [9]. Levels of TNF-α are significantly decreased when SMI-6877002 is properly administered [9, 10]. To establish the proper treatment regimen of SMI-6877002 (Fig. 1A), 100 µl of saline containing 5, 10, or 20 µM of SMI-6877002 was subcutaneously injected once weekly in STZ-diabetic mice, and levels of TNF-α were quantified in pg/ml by ELISA per manufacturer’s instructions (R&D). SMI-6877002 toxicity was defined by lethargy, body weight, respiratory stress, autopsy organ appearance, and mortality rate.

SMI-6877002 treatment of Muller glia

Human Muller glia were obtained from human cadavers (Everight). The posterior section of the retinal globes were mechanically disrupted and incubated in DMEM/HAM F12 media at 37°C with 5% CO2 for 2 weeks. Cell purity of > 95% GLAST+/Vimentin+ Muller glia was confirmed by flow cytometry. Muller glia were incubated with SMI-6877002 2 h prior to a 100 ng/ml stimulation of recombinant IL-17A for 18 h. Supernatants were collected for TNF-α and VEGF ELISA analysis.

RESULTS AND DISCUSSION

Pericyte quantification

Pericyte ghosts were counted in the above-mentioned capillary beds. Alternatively, two pooled retinas per mouse (n = 3/group) were digested in papain ( Worthington) and then collagenase (80 U/ml Sigma Aldrich) to collect cells. Cells were stained with PE-conjugated, anti-mouse PDGFRβ (Abcam) antibody for flow cytometry analysis (C6 Accuri flow cytometer). Gates were set to an isotype control, and PDGFRβ+ pericytes were quantified.

Statistical analysis

Prism software (Graph pad) was used to conduct two-way ANOVA and unpaired t-test with Tukey’s post-hoc analysis, p-values < 0.05 are marked as significant.

CD40-TRAF6 complex. This halts NFκB activation and reduces inflammation [9]. Levels of TNF-α are significantly decreased when SMI-6877002 is properly administered [9, 10]. To establish the proper treatment regimen of SMI-6877002 (Fig. 1A), 100 µl of saline containing 5, 10, or 20 µM of SMI-6877002 was subcutaneously injected once weekly in STZ-diabetic mice, and levels of TNF-α were quantified in pg/ml by ELISA per manufacturer’s instructions (R&D). SMI-6877002 toxicity was defined by lethargy, body weight, respiratory stress, autopsy organ appearance, and mortality rate.

SMI-6877002 treatment of Muller glia

Human Muller glia were obtained from human cadavers (Everight). The posterior section of the retinal globes were mechanically disrupted and incubated in DMEM/HAM F12 media at 37°C with 5% CO2 for 2 weeks. Cell purity of > 95% GLAST+/Vimentin+ Muller glia was confirmed by flow cytometry. Muller glia were incubated with SMI-6877002 2 h prior to a 100 ng/ml stimulation of recombinant IL-17A for 18 h. Supernatants were collected for TNF-α and VEGF ELISA analysis.

Retinal vascular leakage

Retinal vascular leakage was determined as previously described [2, 12]. FITC-BSA (100 µg/gram body weight) was intravenously injected, circulated for 20 min, and retinas (n = 7/group) collected, fixed, and mounted in OCT. Sections were analyzed via fluorescent microscopy and fluorescent intensity was determined using Metamorph Imaging Software (Molecular Devices). Plasma levels of FITC-BSA were used to normalize fluorescence between individual animals.

Capillary degeneration in retina

Acellular capillaries were counted in five fields per retina, as previously described [12–15]. Encuclated eyes were fixed, digested in elastase for 2 h, and immersed in Tris buffer (pH—8.5) for 16 h at 37°C. Capillary beds were extracted by mechanical disruption, stained, (hematoxylin and periodic acid-Schiff) and viewed at 200x magnification on brightfield microscope.

Vascular leakage is one of the earliest clinical symptoms of diabetic retinopathy detected in diabetics, and in this murine model 8-months post-diabetes [17–20]. Vascular leakage is indicated by diffuse hyper-fluorescence in the outer nuclear layer (ONL), outer plexiform layer (OPL), and the inner nuclear layer (INL) of the retina, as highlighted with two red boxes in leakage areas (Fig. 2B). Quantification of vascular leakage, using fluorescent intensity units (FIU), was significantly increased in all retina layers of the untreated diabetic mice than all non-diabetic mice. While retinal vascular leakage was significantly decreased in the SMI-6877002 treated diabetic mice to similar levels of non-diabetic mice (Fig. 2C). This indicates that the CD40-TRAF6 complex plays a pivotal role in retinal vascular leakage.

In non-proliferative diabetic retinopathy and in this 8-month STZ-diabetes model, retinal capillaries degenerate and vascular cells die [12, 20]. Capillary degeneration was examined in the retinal capillary beds (n = 7/group) of untreated and SMI-treated non-diabetic and diabetic mice, by counting the number of acellular capillaries (highlighted by black arrows in the upper panel of Fig. 2E). The number of acellular capillaries in the retinas of untreated diabetic mice was significantly higher than all non-diabetic mice.
Fig. 1  Inflammation in Muller glia and diabetic mice receiving SMI-6877002 treatments. A Schematic of diabetic retinopathy murine model and SMI-6877002 treatment regimen. Quantifications of TNF-α (B) and VEGF (C) in supernatants of unstimulated (white), or IL-17A stimulated human Muller glia that were untreated (black), received 5 μM (light grey), 10 μM (mid-grey), or 20 μM (dark grey) of SMI-6877002 (n = 6/group). Levels of TNF-α (D) and VEGF (E) in retinas (n = 3/group), and hemoglobin A1c (F) and body weight (G) of non-diabetic (ND) and STZ-diabetic (DB) mice that received no treatment (white), 5 μM (light grey), 10 μM (dark grey), or 20 μM (black) of SMI-6877002 injections 1 time a week; 2-months after diabetes was confirmed. Error bars represent the SEM, * = p < 0.01; all p-values were equated using two-way ANOVA and unpaired student’s t-test. Data are representative of 2 separate experiments.
Further, there was no significant difference in acellular capillaries between the SMI-6877002 treated diabetic mice and any of the non-diabetic mice (Fig. 2D). Additionally, the number of pericyte ghosts in these capillary beds were quantified (highlighted by red arrow in the lower panel of Fig. 2E). There was a significantly higher number of pericyte ghosts in the capillaries of untreated diabetic mice than all non-diabetic mice and the SMI-6877002 treated diabetic mice (Fig. 2F). Conversely, the number of viable PDGFR\(\beta^+\) pericytes were quantified in the retinas (\(n = 3\) samples/}

### Table 1: Blood Glucose and Body Weight

| Group           | % HbA1c (Week 30) | Body Weight (Week 30) |
|-----------------|------------------|-----------------------|
| C57BL/6-ND      | 4.8 ± 1.1        | 41.7 ± 6.6            |
| C57BL/6-DB      | 12.7 ± 2.9*      | 28.6 ± 2.3*           |
| +SMI-6877002-ND | 4.9 ± 0.5        | 40.9 ± 2.2            |
| +SMI-6877002-DB | 12.1 ± 2.1*      | 29.1 ± 1.7*           |
Fig. 2 Vascular leakage, capillary degeneration, and pericyte death in the retinas of diabetic mice. A Clinical data of hemoglobin A1c and body weight of mice 30-weeks after diabetes was confirmed. Treated mice received 30 injections of 20 μM SMI-687702. B Representative fluorescent microscopy of vascular leakage (highlighted by two red boxes in leakage areas) in retinal cross-sections of outer nuclear layer (ONL), outer plexiform layer (OPL), and inner nuclear layer (INL) in untreated and treated non-diabetic and diabetic mice. Quantification of vascular leakage (C), and acellular capillaries (D) in each retina of untreated non-diabetic (light grey squares), untreated diabetic (light grey circles), treated non-diabetic (black squares), and treated diabetic (black circles) mice. Scale bars of images = 25 μm. Each data point represents an individual retina from 7 different mice; 8–months post-diabetes. E Representative images of acellular capillaries (upper panel; 5 acellular capillaries in C57BL/6 D8 and 1 acellular capillary in SMI-687702 treated D8 mice are highlighted), and pericyte ghosts (lower panel) in the retinal capillary beds of untreated and treated non-diabetic and diabetic mice. Black arrows highlight acellular capillaries (upper panel) and red arrows highlight pericyte ghosts (lower panel). Scale bars of images = 10 μm. F Quantiﬁcation of pericyte ghosts in retinal capillary beds (n = 5/group). G Quantiﬁcation of PDGF-Bβ pericytes in total retina per ﬂow cytometry analysis (n = 3/group). Error bars represent the SEM, and p-values were equated by two-way ANOVA analysis and unpaired t-test with Tukey’s post-hoc analysis. Data are representative of 2 experiments.

DATA AVAILABILITY
The datasets generated during and/or analyzed during the current study are available from the corresponding author on reasonable request.

REFERENCES
1. Yao JW, Roger SL, Kawasaki R, Lamoureux EL, Kowalski JW, Bek T, et al. Global prevalence and major risk factors of diabetic retinopathy. Diabetes Care. 2012;35:556–64.
2. Sigurdardottir S, Zapadka TE, Lindstrom SI, Liu H, Taylor BE, Lee CA, et al. Diabetes-mediated IL-17A enhances retinal inﬂammation, oxidative stress, and vascular permeability. Cell Immunol. 2019; https://doi.org/10.1016/j.cellimm.2019.04.009
3. Song X, He X, Li X, Qian Y. The roles and functional mechanisms of interleukin-17A family cytokines in mucosal immunity. Cell Mol Immunol. 2016;13:418–31.
4. Abdel-Moneim A, Bakery HH, Allam G. The potential role of IL-17/Th17 cells in both type 1 and type 2 diabetes mellitus. Biomed Pharmacother. 2018;101:287–92.
5. Lindstrom SI, Sigurdardottir S, Zapadka TE, Tang J, Liu H, Taylor BE, et al. Diabetes induces IL-17A-Act1-FADD-dependent retinal endothelial cell death and capillary degeneration. J Diabetes Complications. 2019;33:668–74.
6. Swaidani, S., Liu, C., Zhao, J., Bulek, K., Li, X. TRAF regulation of IL-17 cytokine signaling. Front Immunol. 2019; https://doi.org/10.3389/fimmu.2019.01293
7. Qiu, AW, Bian, Z., Mao, PA, Liu, QH IL-17A exacerbates diabetic retinopathy by impairing Muller cell function via Act1 signaling. Exp Mol Med. 2016; https://doi.org/10.1038/emm.2016.117
8. Subauste, CS CD40, a novel inducer of purinergic signaling: implications to the pathogenesis of experimental diabetic retinopathy. Vision. 2017; https://doi.org/10.3390/vision10030020
9. Chatziegiorgiou A, Seijikens T, Zarzycka B, Engel D, Poggi M, van den Berg S, et al. Blocking CD40-TRAFl signaling is a therapeutic target in obesity-associated insulin resistance. PNAS. 2014;111:2666–91.
10. Van den Berg SM, Seijikens TTP, Kusters EJH, Zarzycka B, Beckers L, den Toom M, et al. Blocking CD40-TRAFl interactions by small molecule inhibitor 6867066 ameliorates the complications of diet-induced obesity in mice. Int J Obes. 2015;39:782–90.
11. Feng, S., Yu, H., Yu, Y., Geng, Y., Li, D., Yang, C., et al. Levels of inflammatory cytokines IL-1β, IL-6, IL-8, IL-17A, and TNF-α in aqueous humor of patients with diabetic retinopathy. J Diabetes Res. 2018; https://doi.org/10.1155/2018/8546423
12. Veenstra A, Liu H, Lee CA, Du Y, Tang J, Kern TS. Diabetic Retinopathy: retina-specific methods for maintenance of diabetic rodents and evaluation of vascular histopathology and molecular abnormalities. Curr Protoc Mouse Biol. 2015;5:247–70.
13. Zapadka TE, Lindstrom SI, Taylor BE, Lee CA, Tang J, Taylor ZRR, et al. RORgamma-T101 lowalt rels retinal inﬁammation, capillary degeneration, and the progression of diabetic retinopathy. Int J Mol Sci. 2020; https://doi.org/10.3390/ijms221103547
14. Zapadka TE, Lindstrom SI, Batoji JC, Liu H, Taylor BE, Howell, SJ, et al. Aryl hydrocarbon receptor agonist-VAF347 impedes retinal pathogenesis in diabetic mice. Int J Mol Sci. 2021; https://doi.org/10.3390/ijms22094335
15. Howell SJ, Lee CA, Batoji JC, Zapadka TE, Lindstrom SI, Taylor BE, et al. Retinal inﬂammation, oxidative stress, and vascular impairment is ablated in diabetic mice receiving XMD8-92 treatment. Front Pharmacol. 2021; https://doi.org/10.3389/fphar.2021.732630
16. Semeran K, Pawloski P, Lisowski L, Szczepaniak I, Wojtowicz J, Lawicki S, et al. Plasma levels of IL-17, VEGF, and adrenomedullin and S-cove dysfunction of the retina in children and adolescents without signs of retinopathy and with varied duration of diabetes. Mediators Inflamm. 2013; https://doi.org/10.1155/2013/274726
17. Kern TS, Tang J, Berkowitz BA. Validation of structural and functional lesions of diabetic retinopathy in mice. Mol Vis. 2010;16:1211–31.
18. Liu H, Tang J, Du Y, Saadane A, Tonade D, Samuels I, et al. Photoreceptor cells influence retinal vascular degeneration in mouse models of retinal degeneration and diabetes. Invest Ophthalmol Vis Sci. 2016;57:4272–81.
19. Antonetti DA, Barber AJ, Khan S, Lieh T, Tarbell JM, Gardner TW, et al. Vascular permeability in experimental diabetes is associated with reduced endothelial occludin content: vascular endothelial growth factor decreases occludin in retinal endothelial cells. Penn State Retina Research Group. Diabetes. 1998;47:1953–9.
20. Bresnick GH, Davis MD, Myers FL, de Venecia G. Clinopathologic correlations in diabetic retinopathy. II. Clinical and histologic appearances of retinal capillary microaneurysms. Arch Ophthalmol. 1977;95:1215–20.

ACKNOWLEDGEMENTS
We would like to thank Dawn Smith, John Denker, Heather Butler, Maryanne Pendergast, Denice Major, and Catherine Doller for outstanding technical assistance. This research was funded by the following grants: NEI-R01 EY030487, VA-101 CX002204, NEI-P30 EY011373, and the Cleveland Eye Bank Foundation.

AUTHOR CONTRIBUTIONS
Each author has made substantial contributions to this study. The following is each author’s contributions: SJH, CAL, and PRT designed the experimental project. SJH, CAL, TEZ, SIL, BET, ZRRT, KGB, and PRT acquired, analysed, and interpreted the data. SJH, CAL, and PRT wrote, reviewed, and edited the manuscript. PRT supervised and provided funding. Finally, all authors have read and approved the submitted version of this manuscript.

COMPETING INTERESTS
The authors declare no competing interests.

ADDITIONAL INFORMATION
Correspondence and requests for materials should be addressed to Patricia R. Taylor.

Reprints and permission information is available at http://www.nature.com/reprints

Nutrition and Diabetes (2022) 12:46
