Ligation of CD40 in Human Müller Cells Induces P2X$_7$ Receptor–Dependent Death of Retinal Endothelial Cells

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Purpose. Cluster of differentiation 40 (CD40) is required for retinal capillary degeneration in diabetic mice, a process mediated by the retinal endothelial cells (REC) death. However, CD40 activates prosurvival signals in endothelial cells. The purpose of this study was to identify a mechanism by which CD40 triggers programmed cell death (PCD) of RECs and address this paradox.

Methods. Human RECs and Müller cells were incubated with CD154 and L-N6-(1-Iminoethyl)lysine (L-Nil, nitric oxide synthase 2 inhibitor), α-lipoic acid (inhibitor of oxidative stress), anti-Fas ligand antibody, or A-438079 (P2X$_7$ adenosine triphosphate [ATP] receptor inhibitor). Programmed cell death was analyzed by fluorescence-activated cell sorting (FACS) or Hoechst/propidium iodide staining. Release of ATP was measured using a luciferase-based assay. Mice were made diabetic with streptozotocin. Expression of P2X$_7$ was assessed by FACS, quantitative PCR, or immunohistochemistry.

Results. Ligation of CD40 in primary RECs did not induce PCD. In contrast, in the presence of primary CD40$^+$ Müller cells, CD40 stimulation caused PCD of RECs that was not impaired by L-Nil, α-lipoic acid, or anti-Fas ligand antibody. We found CD40 did not trigger TNF-α or IL-1β secretion. Primary Müller cells released extracellular ATP in response to CD40 ligation. Inhibition of P2X$_7$ (A-438079) impaired PCD of RECs; CD40 upregulated P2X$_7$ in RECs, making them susceptible to ATP/P2X$_7$–mediated PCD. Diabetic mice upregulated P2X$_7$ in the retina and RECs in a CD40-dependent manner.

Conclusions. Cluster of differentiation 40 induces PCD of RECs through a dual mechanism: ATP release by Müller cells and P2X$_7$ upregulation in RECs. These findings are likely of in vivo relevance since CD40 upregulates P2X$_7$ in RECs in diabetic mice and CD40 is known to be required for retinal capillary degeneration.

Keywords: retinal endothelial cells, Müller cells, purinergic

Degenerate capillaries are a hallmark of early diabetic retinopathy.[1] They are the result of loss of retinal endothelial cells (RECs) and pericytes with the ensuing transformation into tubes of basement membrane that lack blood flow. The resulting ischemia likely promotes the transition to the proliferative form of diabetic retinopathy characterized by retinal neovascularization.

The development of capillary degeneration is a slow process.[1,2] It takes 8 months of diabetes for degenerate capillaries to become apparent in streptozotocin-treated rats.[2] Many studies revealed that RECs undergo programmed cell death (PCD) in the diabetic retina.[1–6] The fact that only a small fraction of RECs have detectable evidence of PCD at a given time is in keeping with a low rate of cell loss that will eventually result in capillary dropout.

The pathogenesis of retinal capillary degeneration in diabetes has not been fully elucidated, and various mechanisms may contribute to this phenomenon. The increased oxidative and nitrosative stress in the diabetic retina have been linked to PCD of RECs and capillary degeneration.[7–11] Tumor necrosis factor (TNF)-α and IL-1 increase activation of caspases in RECs, and the genetic or pharmacologic inhibition of IL-1 or TNF-α signaling diminishes capillary degeneration in diabetic mice.[3,12–14]

Cluster of differentiation 40 (CD40) is a member of the TNF receptor superfamily that has low-level basal expression in retinal Müller cells, RECs, microglia/macrophage, and ganglion neurons.[15] However, CD40 expression in Müller cells, RECs, and microglia/macrophages is upregulated in the diabetic retina.[15] Moreover, CD40 plays a central role in the develop-

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ment of early diabetic retinopathy since diabetic CD40$^{-/-}$ mice are protected from capillary degeneration.[15]

Delineation of the role of CD40 in retinal capillary degeneration requires examining whether CD40 stimulation causes death of RECs. However, ligation of CD40 in endothelial cells does not typically induce cell death,[16–18] likely because CD40 activates PI3K/Akt-mediated prosurvival signals.[17] This raised the possibility that CD40 might promote death of RECs by acting through other retinal cells. We examined Müller cells since they are considered key in the pathogenesis of diabetic retinopathy and these cells encircle RECs. We report that while ligation of CD40 in primary human RECs did not increase PCD, CD40 stimulation promoted PCD of RECs when RECs were incubated with human Müller cells. CD40 triggered ATP release by primary Müller cells, upregulated $P2X_7$ expression in RECs, and caused $P2X_7$-dependent PCD of REC.

Materials and Methods

Cells

Primary human RECs and Müller cells were obtained as described.[15] Retinal endothelial cells were cultured in gelatin-coated flasks containing DMEM plus 10% FBS (HyClone; GE Healthcare Life Sciences, Logan, UT, USA), endothelial cell growth supplement from bovine pituitary (15 μg/mL; Sigma-Aldrich Corp., St Louis, MO, USA) and insulin/transferrin/selenium (Sigma-Aldrich Corp.). Cell identity was confirmed by incorporation of acetylated low-density lipoprotein (>90%). Primary human Müller cells were cultured in DMEM/F12 containing 20% FBS. Cultures were >95% pure for Müller cells (vimentin$^+$, CRALBP$^+$, and GFAP$^-$ by immunofluorescence). Human retinal cells were used between passages 3 to 6. The human Müller cell line MIO-M1 was a gift from Gloria Limb (University College London, London, UK). In coculture experiments, 8×10^4 RECs and 4×10^4 RMCs were cultured per well of 6-well plates.

In Vitro Stimulation

Cells were treated with multimeric human CD154 (CD40 ligand; gift from Richard Kornbluth, Multimeric Biotherapeutics, Inc., La Jolla, CA, USA).[15] As controls we used omission of CD154 or incubation with a nonfunctional CD154 mutant (T147N). We added L-Nil (500 μM; Enzo Life Sciences, Farmingdale, NY, USA); α-lipoic acid (1 mM; Sigma-Aldrich Corp.); A-438079 (10 μM; Tocris Bioscience, Bristol, UK); neutralizing anti-Fas ligand mAb (15 μg/mL; GeneTex, Irvine, CA, USA); or isotype control mAb to cells 1 hour prior to stimulation with CD154. Endothelial cells were also incubated with Bz-ATP (100 μM; Sigma-Aldrich Corp.) during the last 24 hours of incubation with or without CD154, with staurosporine (100 nM; Sigma-Aldrich Corp.) for 6 hours or IFN-γ (100 U/mL; Peprotech, Rocky Hill, NJ, USA) for 72 hours.

Retroviral Vectors and Transductions

Murine stem cell virus-based bicistronic retroviral vector MIEG3 that encodes enhanced green fluorescent protein (EGFP) with or without human CD40 was previously described.[19] Cells were incubated overnight with retrovirus plus polybrene (8 μg/mL, Sigma-Aldrich Corp.). Cells were used at least 72 hours after infection.

Flow Cytometry

Transduced Müller cells were sorted for EGFP expression using a commercial sorter (FACSAria; BD Biosciences, San Jose, CA, USA). Müller cells were >95% EGFP$^+$ when used in experiments. Retinal cells were incubated with an apoptosis detection kit and commercial stain (Annexin V-PE and 7-AAD; BD Biosciences). Unless otherwise stated, staining was performed between 72 to 100 hours after in vitro coculture of RECs and Müller cells. Cells were analyzed on a flow cytometer (LSR II; BD Biosciences). Commercial software (FlowJo; Tree Star, Inc., Ashland, OR, USA) was used for analysis. Cells were incubated with anti-human ICAM-1 (eBiosciences, San Diego, CA, USA); anti-human Fas ligand (BD Biosciences); or isotype-matched control mAbs, or with anti-$P2X_7$ Ab (Alomone Labs, Jerusalem, Israel) plus secondary Ab (eBiosciences). Corrected mean fluorescence intensity was calculated by subtracting mean fluorescence for isotype control or secondary Ab alone from values obtained for specific Abs.

Hoechst 33342 and Propidium Iodide Staining

Retinal endothelial cells were incubated with the blue-fluorescence dye Hoechst 33342 (5 μg/mL) and the red-fluorescence dye propidium iodide (1 μg/mL; both from Molecular Probes; Eugene, OR, USA). Nuclear morphology was examined using a fluorescence microscope (Zeiss Axiovert; Carl Zeiss, Inc., Oberkochen, Germany). Percentages of cells with intense blue fluorescence (nuclear condensation) and cells that stained with propidium iodide (dead cells) were determined.

Measurement of Extracellular ATP

Müller cells were prepared for ATP release assays.[20] The ecto-ATPase inhibitor β, γ-methylene-ATP (300 μM; Sigma-Aldrich Corp.) was added 15 minutes prior to stimulation with CD154. Extracellular ATP was measured in supernatants using an ATP bioluminescence assay kit (Sigma-Aldrich Corp.). Luminescence was quantified using a luminometer (TD 20/20; Turner Designs, San Jose, CA, USA).[20] Concentrations of ATP were calculated using an ATP standard curve.

Cytokine ELISA

Supernatants were used to measure concentrations of human IL-1β and human TNF-α (eBioscience).

Measurement of Nitrite and Superoxide Generation

Nitrite concentrations were calculated using a Griess reaction (Promega Corp., Madison, WI, USA). Superoxide production was examined using the lucigenin assay.[8]

Induction of Diabetes in Mice

Male C57BL/6J, CD40$^{-/-}$ (N° 002928) and P2X7$^{-/-}$ (N° 005576) mice backcrossed to C57BL/6J (Jackson Laboratory, Bar Harbor, ME, USA) were rendered diabetic by administration of streptozotocin (STZ). Fasted mice of 20 to 25 g body weight received five daily intraperitoneal injections of STZ (55 mg/kg; MP Biomedicals, Solon, OH, USA). Development of diabetes (blood glucose >250 mg/mL) was assessed beginning at 1 week. Blood glucose, glycated hemoglobin, and body weights were similar in diabetic B6, CD40$^{-/-}$, and P2X7$^{-/-}$ mice. Studies adhered to the institutional guidelines for humane treatment of animals, "Principles of laboratory animal care' (NIH) and to the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research.

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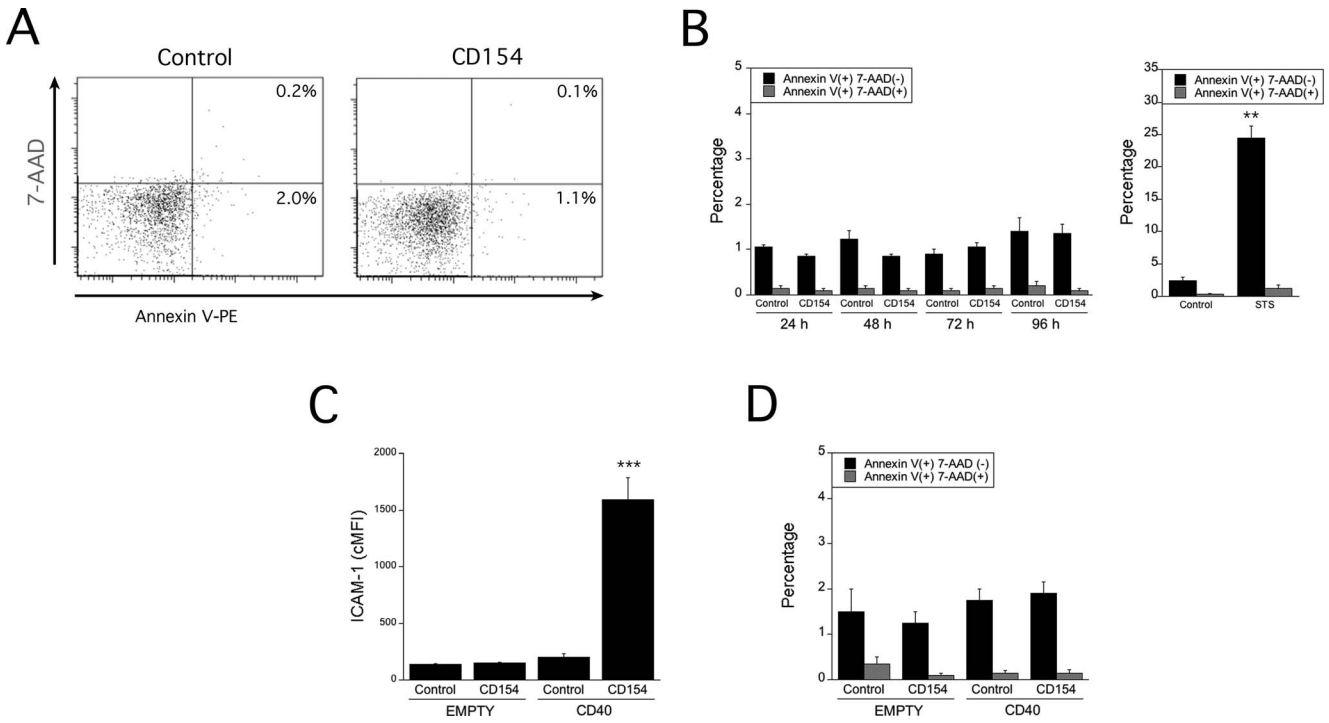

Figure 1. Ligation of CD40 in RECs does not cause PCD. (**A**, **B**) Primary human RECs were incubated with or without CD154 followed by staining with Annexin V or 7-AAD. (**A**) Representative dot plot obtained after 24 hours of incubation with or without CD154. (**B**) We incubated RECs with CD154 as indicated and percentages of cells that stained with Annexin V or 7-AAD were assessed by flow cytometry. Retinal endothelial cells were also incubated with staurosporine for 6 hours. (**C**, **D**) We transduced RECs with an empty retroviral vector (MIEG3) or with a retroviral vector that encodes CD40 (MIEG3-CD40). Cells were incubated with or without CD154. Expression of ICAM-1 (**C**) and percentages of cells that stained with Annexin V or 7-AAD (**D**) were assessed by flow cytometry. Results are representative of three independent experiments. $^{**}P < 0.01$; $^{***}P < 0.001$.

Real-Time Quantitative PCR

Gene expression was assessed using cDNA, SYBR GREEN PCR Master Mix (Applied Biosystems, Foster City, CA, USA), primers for $P2X_7$ receptor[21] or 18S rRNA[22] and a commercial PCR system (7300 Real Time PCR System; Applied Biosystems). Each cDNA sample was run in triplicate. Samples were normalized according to the content of 18S rRNA.

Immunohistochemistry

Paraffin-embedded sections were incubated with Tomato Lectin DyLight 488 (Vector Laboratories, Burlingame, CA, USA) and either anti-P2X7 Ab (Acris, Herford, Germany) or ApopTag Red, In situ Apoptosis Detection kit (EMD Millipore, Billerica, MA, USA). Staining specificity of was confirmed by omitting primary Ab. Retinas were analyzed using an automated microscope (Leica DMI 6000B; Leica Microsystems, Inc., Buffalo Grove, IL, USA) equipped for epifluorescence microscopy.

Statistical Analysis

All results were expressed as the mean ± SEM. Data were analyzed by 2-tailed Student's *t*-test and ANOVA. Differences were considered significant at $P \leq 0.05$.

Results

Ligation of CD40 in Human REC Does Not Result in PCD

Primary RECs incubated with CD154 (CD40 ligand) did not exhibit an increase in the percentages of cells that were Annexin V^+ 7-AAD^- (early apoptosis) or Annexin V^+ 7-AAD^+ (late apoptosis/necrosis; Figs. 1A, 1B). In contrast, staurosporine increased the percentages of Annexin V^+ 7-AAD^- cells (Fig. 1B). Consistent with studies in primary nonhematopoietic cells, human RECs express low levels of CD40 under basal conditions.[15] Thus, to confirm that ligation of CD40 expressed on RECs did not induce PCD, RECs were transduced with an MIEG3-based retroviral vector that encodes human CD40 to increase the percentage of cells that express CD40. This approach allows examining the functional effects of CD40 stimulation in various primary cells including REC.[23,24] CD40 was functional since, as reported,[23] CD154 upregulated ICAM-1 (Fig. 1C). We found CD154 did not increase in the percentages of Annexin V^+ 7-AAD^- cells or Annexin V^+ 7-AAD^+ cells (Fig. 1D). Culturing RECs in normal glucose (5 mmol/L) or high glucose (30 mmol/L) conditions did not affect the results (data not shown). Taken together, CD40 signaling in RECs did not increase PCD.

CD40 Stimulation Results in PCD of Human RECs When Incubated With Müller Cells

We examined whether CD40 induces death of RECs through an indirect mechanism. Given that Müller cells closely associate with RECs, we examined whether CD40 stimulation affected REC survival when these cells were incubated with Müller cells. Müller cells upregulate CD40 in diabetic mice.[15] However, similar to RECs, Müller cells cultured in vitro have minimal levels of CD40 expression under basal conditions.[15] Thus, to test the effect of CD40 ligation in Müller cells, CD40 expression in Müller cells was induced with a retroviral vector.[23,24] Human Müller cells were transduced with MIEG3 or MIEG3-CD40 retroviral vectors. Expression of CD40 induced by retrovirus was similar to that induced by culturing cells in

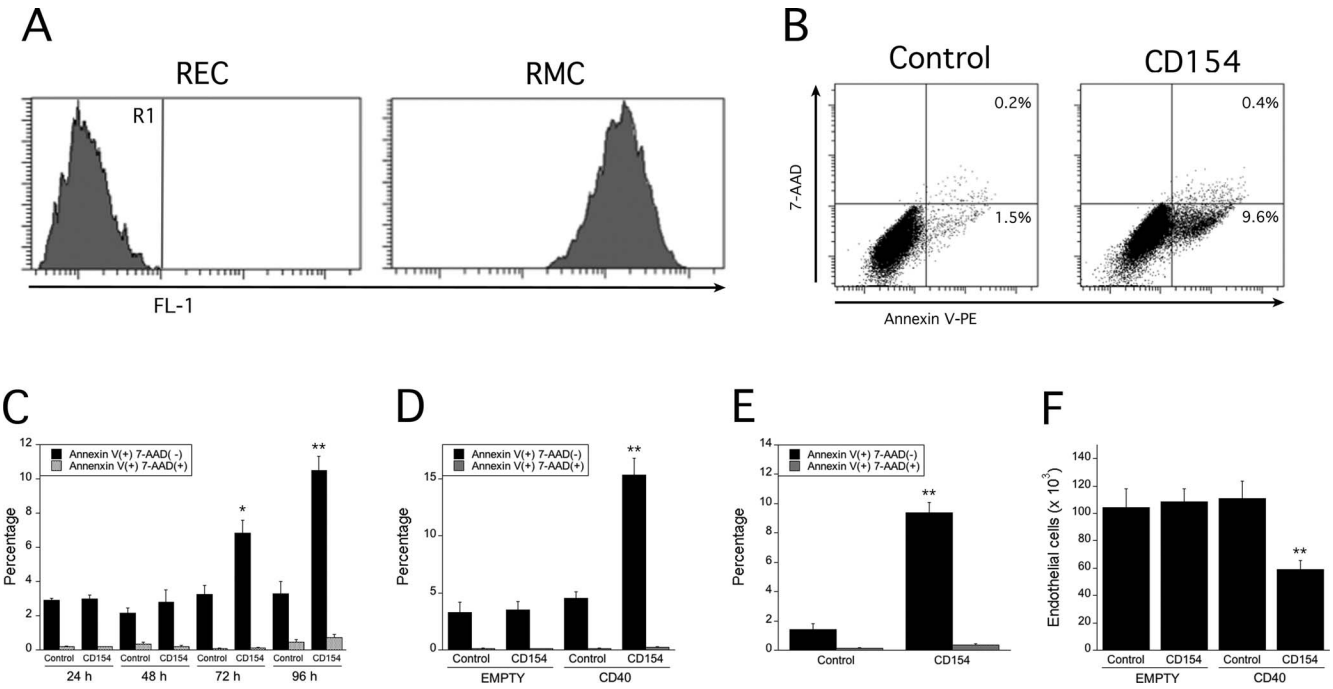

Figure 2. Stimulation of CD40 causes PCD of RECs in the presence of CD40$^+$ Müller cells. (**A**) Green fluorescence of untransduced RECs, Müller cells transduced with retroviral vector that encodes CD40 plus EGFP (MIEG3-CD40). Green fluorescence of Müller cells transduced with empty retroviral vector was similar to that of Müller cells transduced with CD40-encoding retroviral vector (not shown). (**B**, **C**) Primary Müller cells transduced with retroviral vector that encodes CD40 were incubated with RECs in the presence or absence of CD154. Percentages of cells that stained with Annexin V or 7-AAD were assessed by flow cytometry on gated EGFP$^-$ cells (REC). Dot plots show representative data obtained at 96 hours of culture. (**D**, **E**) Primary Müller cells transduced with MIEG3 or with MIEG3-CD40 (**D**) or MIO M1 Müller cells transduced with MIEG3-CD40 (**E**) were incubated with RECs in the presence or absence of CD154 for 96 hours. The percentage of RECs (EGFP$^-$) that stained with Annexin V or 7-AAD was assessed by flow cytometry. (**F**) Primary Müller cells transduced with empty retroviral vector or with retroviral vector that encodes CD40 were incubated with RECs in the presence or absence of CD154. The number of RECs (EGFP$^-$) was determined at 120 hours. Results are representative of 3 to 4 independent experiments. $^*P < 0.05$; $^{**}P < 0.01$.

conditions relevant to diabetes (Portillo et al., Manuscript in preparation). These vectors encode EGFP. Müller cells sorted for EGFP (>95% EGFP$^+$) were incubated with primary RECs. These two populations were distinguished by EGFP expression (Fig. 2A). Expression of Annexin V and 7-AAD were examined in gated EGFP$^-$ (endothelial cells). CD154 increased the percentages of Annexin V$^+$ 7-AAD$^-$ (Fig. 2B) that became significant after 72 hours of in vitro incubation (Fig. 2C). This effect was driven by CD40 signaling in Müller cells since no significant change in Annexin V$^+$ 7-AAD$^-$ cells was detected in cultures that contained Müller cells that were largely CD40$^-$ (Fig. 2D). Similar results were obtained with a human Müller cells line (MIO-M1) transduced with the CD40-encoding vector (Fig. 2E). Moreover, the number of endothelial cells decreased when they were incubated with CD40$^+$ Müller cells plus CD154 (Fig. 2F). Thus, CD40 stimulation increases PCD of RECs in the presence of retinal Müller cells.

PCD of Human RECs Does Not Appear to be Mediated by NOS2, Oxidative Stress, Fas Ligand, TNF-α, or IL-1β

Nitrosative stress has been reported to cause PCD.[7,11] Ligation of CD40 increases nitric oxide production by mouse Müller cells.[15] The coculture of human Müller cells and RECs were incubated with L-Nil, a potent and specific inhibitor of nitric oxide (NO) production, followed by addition of CD154. We found L-NIL did not impair the induction of Annexin V$^+$ 7-AAD$^-$ RECs in CD40-activated cell cultures (Fig. 3A). Moreover, although CD40 ligation upregulates nitric oxide synthase (NOS2) in human Müller cells,[15] these cells did not exhibit detectable secretion of nitric oxide when incubated with CD154 for up to 72 hours (<0.39 μM; not shown). Oxidative stress has been implicated in the development of capillary degeneration in diabetic retinopathy.[8–10] In vivo administration of the antioxidant α-lipoic acid diminishes PCD of RECs in diabetic rats.[9] However, incubation with α-lipoic acid failed to impair the increase in Annexin V$^+$ 7-AAD$^-$ endothelial cells in cultures stimulated with CD154 (Fig. 3B). Regardless of duration of incubation, Müller cells did not increase superoxide production in response to CD154 (control: 116.6 ± 15.6; CD154: 103.3 ± 7.9 relative units/mg protein; $P = 0.4$). TNF-α and IL-1β stimulate cell death signaling in REC.[12] Nonetheless, CD154 did not induce TNF-α or IL-1β production by Müller cells or RECs (<3.9 pg/mL; not shown). Next, we examined whether CD154 induced PCD of RECs through Fas ligand-Fas interaction. Addition of a blocking anti-Fas ligand antibody failed to affect the induction of Annexin V$^+$ and 7-AAD$^-$ RECs (Fig. 3C). Moreover, Müller cells remained Fas Ligand negative regardless of duration of incubation with CD154 (not shown). Thus, NOS2, oxidative stress, TNF-α, IL-1β and Fas ligand do not appear to mediate CD40-mediated PCD of RECs.

Inhibition of the ATP Receptor P2X$_7$ Prevents PCD of RECs

Extracellular ATP can induce apoptosis or necrosis through binding to the purinergic receptor P2X$_7$.[25–28] We examined whether CD40 ligation in primary Müller cells induces release of extracellular ATP. As shown in Figure 4A, Müller cells that expressed CD40 secreted extracellular ATP in response to CD154. Next, the selective P2X$_7$ receptor inhibitor A-

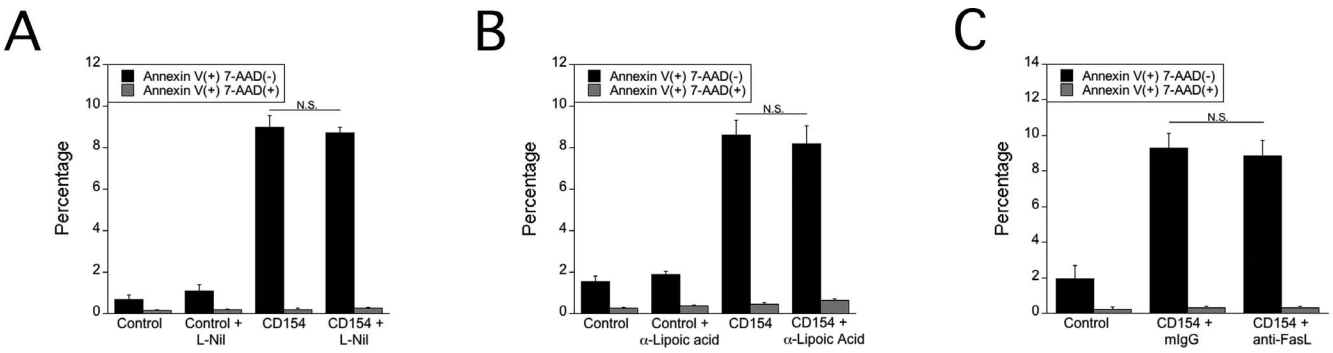

Figure 3. Programmed cell death of RECs is not prevented by an NOS2 inhibitor, an antioxidant, or an anti-Fas ligand mAb. Primary Müller cells transduced with a retroviral vector that encodes CD40 were incubated with RECs. The nitric oxide synthase inhibitor L-NIL (**A**), antioxidant α-lipoic acid (**B**) and a blocking anti-Fas ligand mAb (**C**) were added prior to stimulation with CD154. Percentage of RECs (EGFP$^-$) that stained with Annexin V or 7-AAD was assessed by flow cytometry. Results are representative of three independent experiments.

438079[29,30] was added to the coculture of RECs and Müller cells stimulated with CD154. $P2X_7$ receptor inhibition prevented the increase in Annexin V$^+$ 7-AAD$^-$ RECs in cultures stimulated with CD154 (Fig. 4B). Thus, CD40 ligation induces ATP release by primary Müller cells and triggers PCD of bystander RECs that appears dependent on $P2X_7$.

CD40 Ligation Upregulates $P2X_7$ Receptor Expression in RECs and Increases Their Susceptibility to PCD Triggered by ATP

The susceptibility to $P2X_7$ receptor–mediated PCD varies among cells. Addition of CD154 to cocultures of Müller cells and RECs did not enhance PCD of RECs until after 72 hours of incubation. Given that RECs express CD40 under resting conditions,[15] we examined whether the susceptibility of RECs to ATP-mediated PCD is enhanced over time by incubation with CD154. Retinal endothelial cells were cultured with or without CD154, and Bz-ATP (a P2X purinergic agonist) was added during the last 24 hours of culture. Incubation with CD154 caused a significant increase in the percentages of Annexin V$^+$ 7-AAD$^-$ RECs after addition of Bz-ATP, an effect that was detected after 72 hours of incubation with CD154 (Fig. 5A). Retinal endothelial cells were also stained with Hoechst 33342 and propidium iodide. Increased PCD was confirmed by detecting higher percentages of RECs that stained brightly with Hoechst 33342 (nuclear condensation) and were propidium iodide negative when these cells were incubated with CD154 for 72 hours and exposed to Bz-ATP (Fig. 5B). An increase in $P2X_7$ receptor levels promotes the effects of this receptor.[31] Thus, we examined the effect of CD154 on $P2X_7$ receptor expression. We used IFN-γ as a positive control. Human RECs expressed low levels of $P2X_7$ receptor under basal conditions (Fig. 5C). Incubation with CD154 for ≥72 hours increased $P2X_7$ receptor expression in RECs (Fig. 5C). Taken together, CD40 ligation upregulates $P2X_7$ receptor in RECs and increases the susceptibility of these cells to PCD induced by a $P2X_7$ receptor agonist.

CD40 Promotes $P2X_7$ Receptor Upregulation in the Retina and Retinal Endothelial Cells of Diabetic Mice

A small percentage of RECs undergo PCD in the diabetic retina, a phenomenon that leads to development of capillary degeneration.[1–6] CD40 is required for capillary degeneration in diabetic mice.[15] Indeed, tomato lectin$^+$ (endothelial) cells that were TUNEL$^+$ were noted in the retinas of diabetic B6 mice (1.15 ± 0.21 TUNEL$^+$ tomato lectin$^+$ cells/retina section in diabetic mice versus no TUNEL$^+$ tomato lectin$^+$ cells in nondiabetic controls; $n = 7$; $P < 0.01$), whereas no TUNEL$^+$ tomato lectin$^+$ cells were detected in diabetic or nondiabetic CD40$^{-/-}$ or P2X7$^{-/-}$ mice ($n = 6$). To further assess the relevance of $P2X_7$ receptor, we examined whether expression

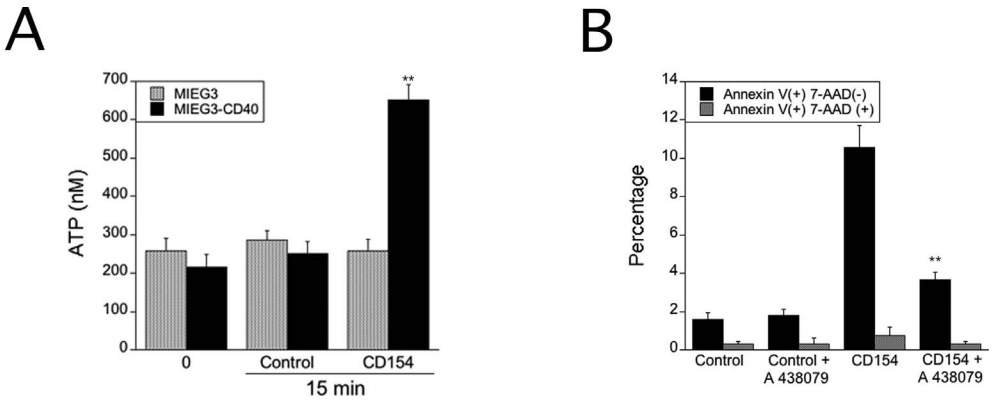

Figure 4. Programmed cell death of RECs is impaired by inhibition of the $P2X_7$ receptor. (**A**) Primary human Müller cells transduced with empty or CD40-encoding MIEG3 retroviral vectors were incubated with CD154. Release of ATP was assessed by a luciferase-based assay. (**B**) Primary Müller cells transduced with a retroviral vector that encodes CD40 were incubated with RECs. We added the $P2X_7$ receptor inhibitor A 438079 prior to stimulation with CD154. The percentage of RECs (EGFP$^-$) that stained with Annexin V or 7-AAD was assessed by flow cytometry. Results are representative of three independent experiments. $^{**}P < 0.01$.

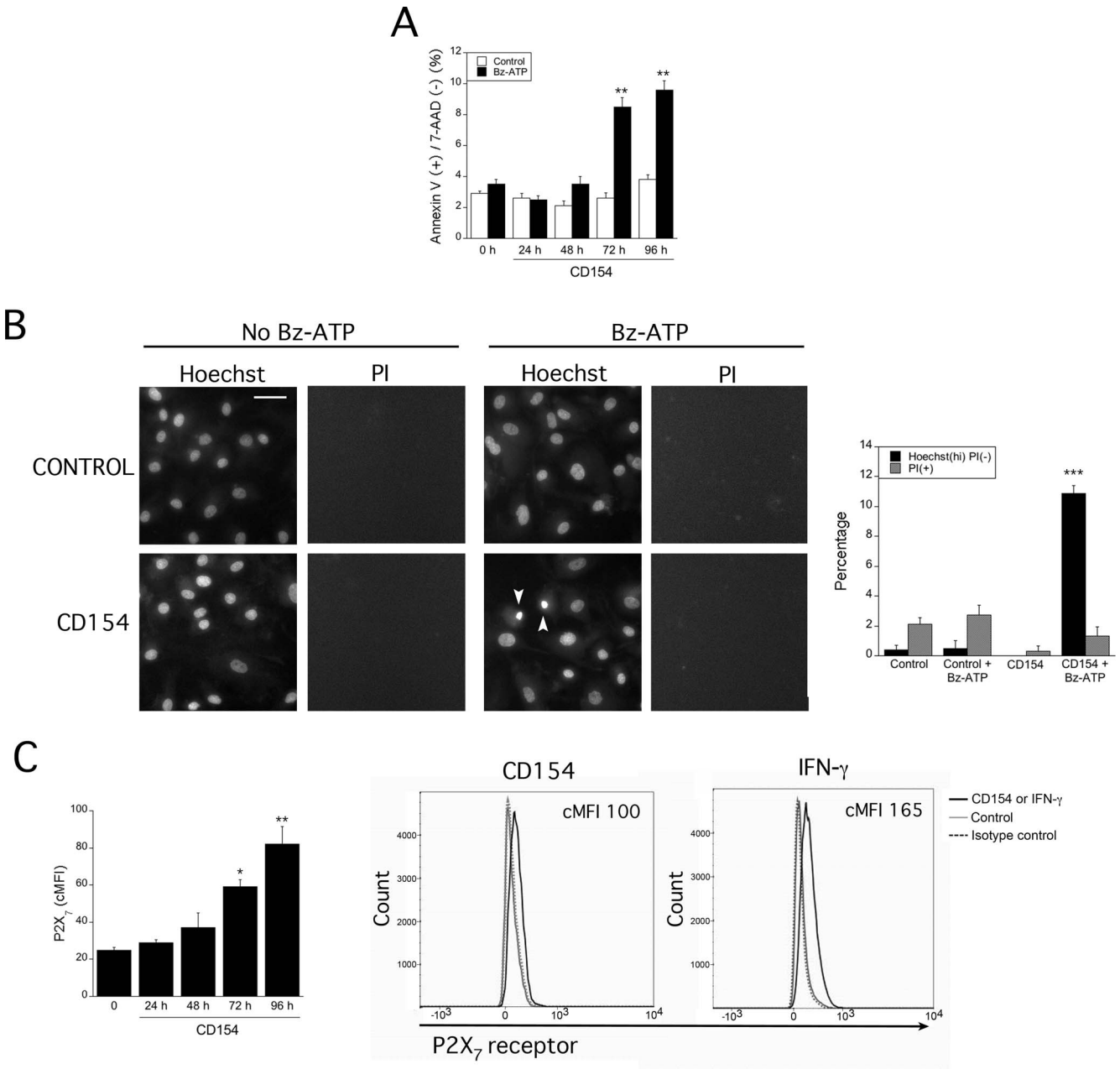

Figure 5. Cluster of differentiation 154 upregulates $P2X_7$ receptor in RECs and increases their susceptibility to ATP-driven PCD. (**A**) We incubated RECs with or without CD154 as indicated, followed with incubation with Bz-ATP (100 μM) for 24 hours. We stained RECs with Annexin V plus 7-AAD. (**B**) We cultured RECs with or without CD154 for 72 hours followed by incubation with or without Bz-ATP for 24 hours and stained with Hoechst 33342 plus propidium iodide. *Arrowheads* show RECs that stained intensely with Hoechst 33342 (nuclear condensation). *Bar*: 20 μm. (**C**) We stimulated RECs with or without CD154 or IFN-γ. Expression of $P2X_7$ receptor in RECs was assessed by flow cytometry. Histograms show $P2X_7$ receptor expression after incubation in complete medium alone (control), CD154 (96 hours) or IFN-γ (72 hours) of in vitro culture. *Dotted line*: secondary Ab alone. *Thin line*: control. *Thick line*: CD154 or IFN-γ. Results are representative of three independent experiments. $^*P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$.

of $P2X_7$ receptor in the retina of diabetic mice is increased in a CD40-dependent manner. Diabetic B6 mice exhibited a marked upregulation of $P2X_7$ receptor mRNA levels (Fig. 6A). This response was largely dependent on CD40 since no significant $P2X_7$ receptor upregulation was detected in diabetic $CD40^{-/-}$ mice. Moreover, diabetic B6 mice exhibited increased $P2X_7$ receptor expression in RECs (Figs. 6B, 6C). Thus, CD40 promotes $P2X_7$ receptor upregulation in the retina and RECs of diabetic mice.

Discussion

Herein we identified a mechanism by which CD40 can promote PCD of primary endothelial cells. We report that, whereas direct CD40 ligation in RECs did not result in PCD, CD40 stimulation enhanced PCD of RECs when these cells were incubated with Müller cells that express CD40. This effect did not appear to be mediated by NOS2, oxidative stress, TNF-α, IL-1β, or Fas ligand. Ligation of CD40 in primary Müller cells increased secretion of extracellular ATP, while CD40

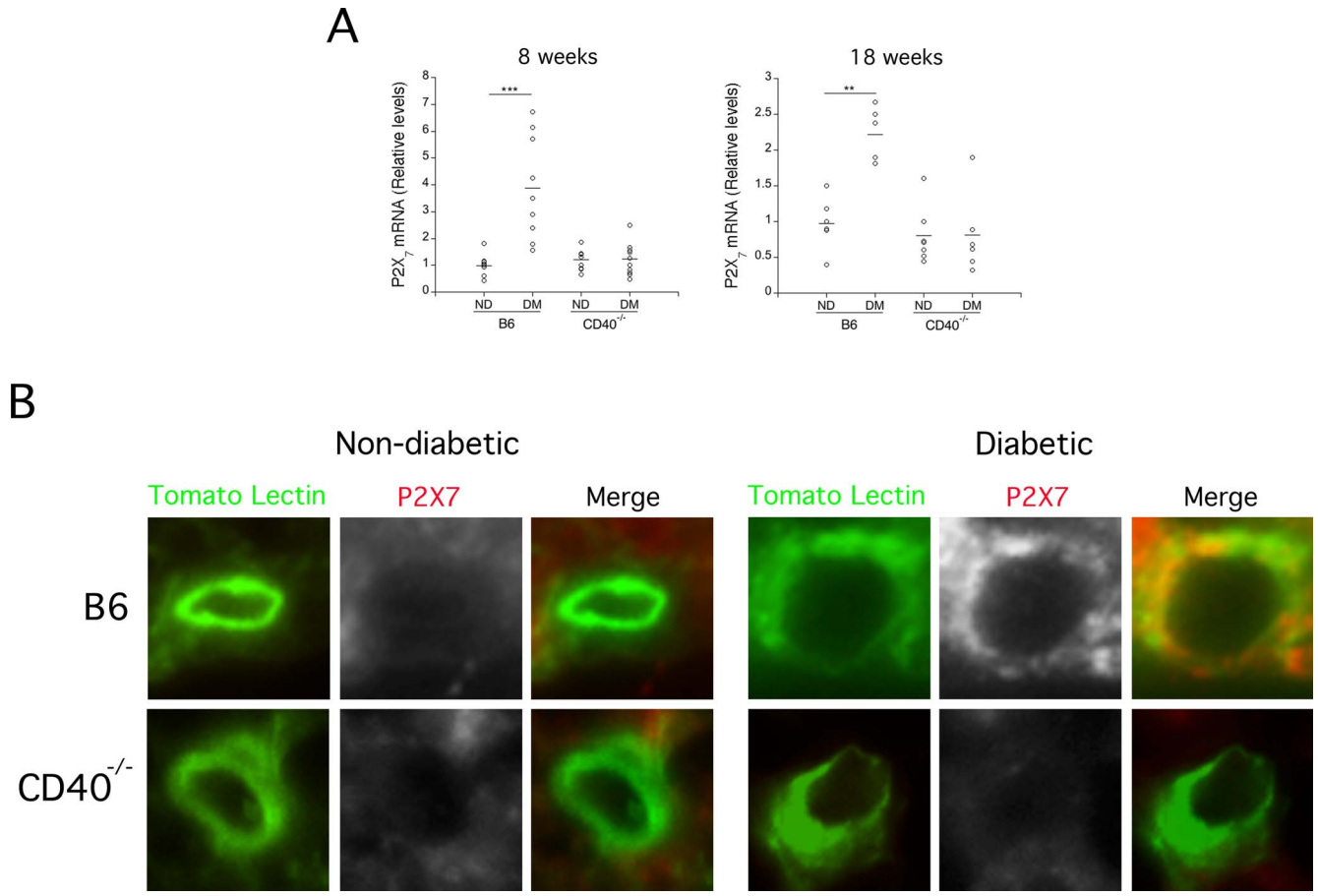

Figure 6. Cluster of differentiation 40 promotes upregulation of $P2X_7$ receptor in the retina and RECs from diabetic mice. (**A**) At 8 and 18 weeks of diabetes, retinas from diabetic B6, $CD40^{-/-}$ as well as from nondiabetic control animals were collected and used for mRNA extraction. We assessed mRNA levels by real-time quantitative PCR using 18S rRNA as internal control. One nondiabetic B6 mouse was given an arbitrary value of 1 and data are expressed as a fold-increase compared with this animal. Data shown represent mean ± SEM (6 to 10 animals per group). $^{**}P < 0.01$; $^{***}P < 0.001$. (**B**) Retinal sections from B6 and $CD40^{-/-}$ mice at 8 weeks of diabetes and from nondiabetic controls were incubated with anti-$P2X_7$ receptor Ab plus tomato lectin to label endothelial cells. Original magnification ×630; 6 to 7 mice/group.

ligation in RECs upregulated $P2X_7$ receptor expression and made them susceptible to ATP-induced PCD. Inhibition of $P2X_7$ receptor during the coculture of RECs and Müller cells inhibited PCD of RECs. These results may be of in vivo relevance since $P2X_7$ is upregulated in a CD40-dependent manner in the retina of diabetic mice, RECs in these animals had increased $P2X_7$ receptor expression and CD40 is known to be required for retinal capillary degeneration.

Extracellular ATP binds two subfamilies of purinergic receptors: the inotropic, ligand-gated P2X receptors and the metabotropic, G protein-coupled P2Y receptors.[32] An important distinction between $P2X_7$ receptor and other P2X receptors is that sustained activation of $P2X_7$ receptor results in formation of transmembrane pores that are permeable to hydrophilic molecules of up to 900 Da.[33–35] Receptor $P2X_7$ mediates apoptotic or necrotic cell death depending on the cell type, duration of incubation with ATP and ATP concentration.[25–28] Several lines of evidence support that CD40 stimulation in Müller cells induced PCD of RECs through $P2X_7$ receptor. First, primary Müller cells secreted extracellular ATP in response to CD40 ligation. Second, CD40 ligation upregulated $P2X_7$ receptor expression in RECs. Third, the $P2X_7$ receptor ligand Bz-ATP induced PCD of CD40-stimulated RECs. Fourth, a selective $P2X_7$ inhibitor prevented PCD of RECs. Addition of extracellular ATP at concentrations of 10 to 100 μM have been reported to be required for induction of PCD.[36,37] The concentrations of extracellular ATP detected in bulk cell-free supernatants from CD40-stimulated Müller cells were lower than these concentrations. However, compared with single administration of extracellular ATP, cell death after repeated exposure to ATP requires lower concentrations of the nucleotide.[35] In addition, luciferase-based assays of ATP concentrations in extracellular medium underestimate the concentrations of ATP in the intercellular space.[38] Assays based on targeting luciferase to the surface of intact cells revealed higher concentrations of ATP at the cell surface level (100-200 μM).[38,39]

Susceptibility to ATP-mediated PCD varies among cell types. Our studies suggest that CD40 plays a dual role in promoting PCD of RECs: it enhances release of extracellular ATP by primary Müller cells and increases the susceptibility of RECs to $P2X_7$-driven PCD that would overcome the prosurvival signals activated by CD40 ligation. Increased susceptibility to PCD may be mediated by CD40-driven upregulation of the $P2X_7$ receptor in RECs since increased $P2X_7$ receptor levels promote the effects of this receptor.[31] However, it is also possible that CD40 ligation may increase susceptibility to $P2X_7$ receptor–mediated PCD through modulation of ATP-gated channel expression, ectoATPase activity, and/or coupling to downstream cell signaling pathways that promote cell death. Of

relevance, diabetes or incubation with high concentrations of glucose appears to increase the susceptibility to ATP-driven PCD in retinal microvessels, fibroblasts, and T cells.[40–42] Thus, CD40 may contribute to increased susceptibility to ATP-mediated PCD that appears to occur in diabetes.

Müller cells become dysfunctional in diabetic retinopathy[43–45] and are likely to affect other retinal cells since Müller cells span the whole width of the retina and provide structural support to retinal blood vessels and neurons. This study, together with the fact that Müller cells surround retinal capillaries, suggests that CD40-driven ATP release may play an important role in the pathogenesis of PCD of RECs and capillary degeneration in diabetic retinopathy. Indeed, expression of CD40 restricted to Müller cells was sufficient to cause capillary degeneration in diabetic transgenic mice.[46] Long-term studies in diabetic $P2X_7^{-/-}$ mice may help elucidate the in vivo role of this receptor in capillary degeneration. We recently observed that $P2X_7$ receptor also promotes expression of proinflammatory cytokines in the diabetic retina.[46] Ligation of CD40 in human and mouse Müller cell lines caused secretion of ATP that in turn induced $P2X_7$ receptor–dependent IL-1β and TNF-α secretion by bystander myeloid cells.[46] Moreover, diabetic transgenic mice that expressed CD40 restricted to Müller cells exhibited retinal upregulation of IL-1β and TNF-α was dependent on $P2X_7$ receptor.[46] These results together with the present study indicate that the CD40-ATP-$P2X_7$ receptor pathway plays an important role in induction of inflammatory responses and death of RECs, events considered pathogenic in the development of diabetic retinopathy.

Our studies centered on Müller cells since CD40 expression restricted to these cells is sufficient to cause capillary degeneration in diabetes. However, it is possible that CD40 signaling in other cell types (including REC) may induce ATP release setting in motion PCD of RECs. In addition, while our studies indicate that CD40-induced ATP release followed by $P2X_7$ receptor signaling in RECs mediates PCD, it is possible that CD40 may also promote death of RECs and capillary degeneration in vivo through additional mechanisms. CD40 enhances retinal leukostasis in diabetic mice, upregulates NOS2, increases retinal protein nitration and upregulates TNF-α and IL-1β in the retinas of diabetic mice.[15,46] All these phenomena have been linked to PCD of RECs and retinal capillary degeneration.[3,7,11–14,47]

In summary, these studies uncovered a mechanism by which CD40 enhances PCD of RECs and suggest that CD40 signaling in Müller cells may be an important contributor to vascular injury in diabetic retinopathy. Our findings may be relevant to other CD40-driven diseases such as atherosclerosis and inflammatory bowel disease. Increased PCD accompanies these disorders and $P2X_7$ receptor has been suggested to be pathogenic in these diseases.[48,49] Strong preclinical data indicate that blockade of the CD40-CD154 pathway can be effective therapy for various inflammatory and neurodegenerative disorders. Our studies suggest that novel approaches to inhibit CD40 signaling may be effective in the management of diabetic retinopathy.

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