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CD40 in Retinal Müller Cells Induces $P2X_7$-Dependent Cytokine Expression in Macrophages/Microglia in Diabetic Mice and Development of Early Experimental Diabetic Retinopathy

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Müller cells and macrophages/microglia are likely important for the development of diabetic retinopathy; however, the interplay between these cells in this disease is not well understood. An inflammatory process is linked to the onset of experimental diabetic retinopathy. CD40 deficiency impairs this process and prevents diabetic retinopathy. Using mice with CD40 expression restricted to Müller cells, we identified a mechanism by which Müller cells trigger proinflammatory cytokine expression in myeloid cells. During diabetes, mice with CD40 expressed in Müller cells upregulated retinal tumor necrosis factor-α (TNF-α), interleukin 1β (IL-1β), intracellular adhesion molecule 1 (ICAM-1), and nitric oxide synthase (NOS2), developed leukostasis and capillary degeneration. However, CD40 did not cause TNF-α or IL-1β secretion in Müller cells. TNF-α was not detected in Müller cells from diabetic mice with $CD40^+$ Müller cells. Rather, TNF-α was upregulated in macrophages/microglia. CD40 ligation in Müller cells triggered phospholipase C–dependent ATP release that caused $P2X_7$-dependent production of TNF-α and IL-1β by macrophages. $P2X_7^{-/-}$ mice and mice treated with a $P2X_7$ inhibitor were protected from diabetes-induced TNF-α, IL-1β, ICAM-1, and NOS2 upregulation. Our studies indicate that CD40 in Müller cells is sufficient to upregulate retinal inflammatory markers and appears to promote experimental diabetic retinopathy and that Müller cells orchestrate inflammatory responses in myeloid cells through a CD40-ATP-$P2X_7$ pathway.

Increasing evidence indicates that chronic low-grade inflammation is important for the development of diabetic retinopathy (1,2). Tumor necrosis factor-α (TNF-α) and interleukin 1β (IL-1β) are proinflammatory molecules upregulated in this disease (3,4). Macrophages/microglia in the diabetic retina express TNF-α (4). Moreover, both cytokines contribute to diabetes-induced degeneration of retinal capillaries, a hallmark of diabetic retinopathy (5,6). In addition to macrophages/microglia, Müller cells (the major retinal macroglia) become dysfunctional in diabetes and contribute to the development of diabetic retinopathy (7). However, little is known about whether Müller cells enhance proinflammatory responses in macrophages/microglia in diabetes.

CD40 is an important driver of retinal inflammation in experimental diabetic retinopathy (8,9). CD40 is upregulated in retinal Müller cells, endothelial cells, and microglia in diabetic mice (8). CD40 ligation in Müller cells and endothelial cells upregulates intracellular adhesion molecule 1 (ICAM-1) and chemokine (C-C motif) ligand 2 (CCL2)

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(8,9). CD40 ligation in monocytes/macrophages/microglia upregulates TNF-α, IL-1β, inducible nitric oxide synthase 2 (NOS2), and CCL2 (10–12). CD40 drives ICAM-1 and CCL2 upregulation, increases protein nitration and the number of leukocytes adherent to blood vessel walls (leukostasis) in the retina of diabetic mice, and is required for the development of capillary degeneration (8,9).

CD40 in hematopoietic cells has been considered central to the development of inflammatory diseases. Although studies using bone marrow chimeras suggest that CD40 expressed in nonhematopoietic cells is also required for inflammation (13), it is not known whether expression of CD40 restricted to the nonhematopoietic compartment is sufficient for development of inflammatory disorders.

Using transgenic mice with expression of CD40 in Müller cells, we report that after induction of diabetes, CD40 expression in these nonhematopoietic cells was sufficient for inflammatory molecule upregulation and development of capillary degeneration. TNF-α was upregulated in macrophages/microglia rather than in Müller cells. CD40 ligation in Müller cells induced macrophages to secrete TNF-α and IL-1β via an ATP-$P2X_7$ receptor pathway. Pharmacologic or genetic inhibition of the $P2X_7$ receptor in diabetic mice impaired not only TNF-α and IL-1β upregulation but also upregulation of ICAM-1 and NOS2, molecules reported to be driven by TNF-α and/or IL-1β. Thus, CD40 in Müller cells orchestrates inflammatory responses in macrophages/microglia and promotes the development of experimental diabetic retinopathy.

RESEARCH DESIGN AND METHODS

Transgenic Mice

Mouse CD40 construct was inserted into the *Eco* RI and *Bam* HI sites of the $pTet^{OS}$ plasmid (14). After sequence verification, the transgene was excised by *Sal* I digestion (14) and microinjected into mouse oocytes (B6). Founder Tet^{OS}-CD40 mice were identified by PCR using the following primers: Tet^{OS}CD40 forward: 5′-GCAACGTGCTGGTTATTGTG-3′, reverse: 5′-CCGGGACTTTAAACCACAGA-3′. The driver line consisted of transgenic mice that express tetracycline (Tet)-repressible transactivator (tTA) under the control of the glial fibrillary acidic protein (GFAP) promoter *gfa2* consisting of the 2.2 kb of 5′-flanking DNA of human GFAP (15). Homozygous Tet^{OS}-CD40 (responder) and heterozygous GFAP-tTA (driver) transgenic mice (15) (both B6) were backcrossed onto a $CD40^{-/-}$ (B6) background. To confirm that transgenic mice were $CD40^{-/-}$, animals were genotyped using primers that detect wild-type CD40 and mutant CD40 (neomycin cassette inserted into exon 3 resulting in lack of functional CD40) (16). Both lines of mice were bred and offspring identified by PCR analysis of genomic DNA. PCR primers for CD40 and tTA were obtained from The Jackson Laboratory. Littermates that inherited only one transgene (single transgenic and nonexpressing) served as controls (Trg-Ctr) for double transgenic animals (Trg-CD40; expressing GFAP promoter-specific CD40 expression).

Induction of Diabetes

Mice were made diabetic using streptozotocin (STZ). Fasted mice (20–25 g body weight) received five daily intraperitoneal injections of STZ (55 mg/kg; MP Biomedicals). Development of diabetes (blood glucose >250 mg/mL) was assessed beginning 1 week after the first injection of STZ. Glycated hemoglobin was measured at 2 months (VARIANT Classic; Bio-Rad). Mice were weighed weekly and, if needed, received insulin to prevent weight loss while maintaining chronic hyperglycemia (target range 350–500 mg/mL). The dose given (0–0.2 units of NPH insulin subcutaneously, 0–3 times per week) was determined individually for each animal. The insulin requirement was similar for all groups of diabetic mice. Studies adhered to the institutional guidelines for humane treatment of animals, *Principles of Laboratory Animal Care* (National Institutes of Health) and to the *Statement for the Use of Animals in Ophthalmic and Visual Research* (Association for Research in Vision and Ophthalmology). Studies were approved by the Case Western Reserve University Institutional Animal Care and Use Committee.

Leukostasis

The number of leukocytes adherent to the retinal microvasculature was determined at 2 months of diabetes. After perfusion with PBS, fluorescein-coupled concanavalin A lectin (20% g/mL; Vector Laboratories) was infused (17). Retinal flat mounts were analyzed using fluorescence microscopy, and brightly fluorescent leukocytes were counted.

Vascular Histopathology

Retinal vasculature was isolated and stained with periodic acid Schiff (8). Eight areas in the mid retina were examined blindly under original magnification ×400. Degenerate capillaries were defined as capillary-sized tubes of material positive for periodic acid Schiff without nuclei along the capillary. Samples were processed blindly.

Immunohistochemistry

Paraffin-embedded eyes fixed with formalin-free zinc fixative (24 h; BD Biosciences) were treated with proteinase K or citrate buffer (8–12 sections per mouse) (8). Antibodies are listed in the Supplementary Data. Retinas were analyzed using a Leica DMI 6000B epifluorescence microscope.

Real-time Quantitative PCR

Real-time PCR was performed as described using primers for ICAM-1 (8), CCL2 (9), TNF-α (18), IL-1β (19), NOS2 (20), $P2X_7$ receptor (21), and 18S rRNA (8).

Flow Cytometry

Retinal cell suspensions were obtained and permeabilized as described (22). Antibodies are listed in the Supplementary Data. CD40 expression is expressed as the corrected mean fluorescence intensity (22).

Cells

Primary human Müller cells obtained as described (8) and the human Müller cell line MIO M1 (gift from Dr. Gloria Limb, University College London, London, U.K.; both >95% vimentin$^+$, cellular retinaldehyde binding protein [CRALBP]$^+$ and

GFAP$^-$) were transduced with a retroviral vector that encodes human CD40 or an empty vector (9). The Müller cell lines mRAST and rMC1 (mouse and rat, respectively) were transduced with a retroviral vector that encodes hmCD40. Müller cells were incubated with multimeric human CD154 to induce CD40 stimulation (obtained from Dr. Richard Kornbluth, Multimeric Biotherapeutics Inc., La Jolla, CA) or with a nonfunctional CD154 mutant (T147N) as the control (13). Cells were also incubated with recombinant FLAG-tagged CD154 (CD40L) plus enhancer (anti-FLAG antibody) or enhancer alone (Enzo Life Sciences). In certain experiments, Müller cells were incubated with BAPTA-AM (1,2-Bis[2-aminophenoxy] ethane-N,N,N′,N′-tetraacetic acid tetrakis[acetoxymethyl ester]; 25 μmol/L; Sigma Aldrich), the phospholipase C inhibitor U73122, or the inactive analog U73343 (1 μmol/L; Tocris Bioscience). Human Müller cells were also cultured with the human monocytic cell line MonoMac6 pretreated with interferon-γ (100 IU for 48 h), with or without the $P2X_7$ receptor inhibitor A-438079 (10 μmol/L; Tocris Bioscience). Mouse Müller cells were incubated with bone marrow-derived macrophages (BMM) from B6 or $P2X_7^{-/-}$ mice plus sodium polyoxotungstate (10 μmol/L) to inhibit the ectonucleotidase CD39 expressed in BMM. MonoMac6 and BMM were pretreated with lipopolysaccharide (100 ng/mL) for 4 h in studies of IL-1β secretion. MonoMac6 cells were transfected with human $P2X_7$ small interfering (si)RNA or control siRNA (Qiagen) using *Trans*IT-X2 (Mirus).

Measurement of Extracellular ATP

The ecto-ATPase inhibitor β,γ-methylene–ATP (300 μmol/L; Sigma-Aldrich) was added 15 min before stimulation with CD154. Extracellular ATP was quantified using an ATP bioluminescence assay kit and an ATP standard curve (Sigma-Aldrich) (23).

Immunoblot

Membranes were probed with antibody to $P2X_7$ receptor, actin (Santa Cruz Biotechnology), total phospholipase Cγ1 (PLCγ1; Cell Signaling), or phospho-Tyr783 PLCγ1 (Cell Signaling).

ELISA

Human IL-1β, human TNF-α, rat TNF-α, mouse IL-1β, and TNF-α (all from eBioscience), human CCL2, mouse CRP (both R&D Systems), and CD154 (Boster Bio) were measured using ELISA.

Statistical Analysis

Results are expressed as the mean ± SEM. Data were analyzed by two-tailed Student *t* test or ANOVA. Differences were considered statistically significant at $P < 0.05$.

RESULTS

CD40 Expression in Müller Cells

We examined the effect of diabetes on serum CD154 (CD40 ligand) levels. Serum CD154 concentrations were significantly increased in mice made diabetic using STZ, whereas CRP levels remained unchanged, and TNF-α was undetectable (<3.9 pg/mL) (Supplementary Fig. 1). Diabetic mice are reported to upregulate CD40 in retinal Müller cells, and diabetic $CD40^{-/-}$ mice are protected from experimental diabetic retinopathy (8). To examine the role of CD40 expressed in Müller cells in experimental diabetic retinopathy, we used a binary transgenic mouse system to obtain $CD40^{-/-}$ mice where CD40 expression is rescued in retinal Müller cells. The driver line consisted of mice expressing the tTA under the control of the human GFAP promoter *gfa2* (GFAP-tTA mice). These mice (B6 background) were backcrossed with $CD40^{-/-}$ mice (also B6 background). The responder line consisted of homozygous $CD40^{-/-}$ mice containing mouse CD40 cloned downstream of the Tet^{OS} promoter (Supplementary Fig. 2*A*). After mating GFAP-tTA mice with Tet^{OS} CD40 animals, double-transgenic offspring (Trg-CD40) are predicted to exhibit rescued CD40 expression in retinal macroglia. In contrast, no such rescue should occur in single-transgenic mice carrying the GFAP-tTA or Tet^{OS} CD40. PCR analysis of genomic DNA identified Trg-Ctr or Trg-CD40 mice (Supplementary Fig. 2*B*). Trg-CD40 mice expressed CD40 in vimentin$^+$ cells (Müller cells), whereas Trg-Ctr animals lacked CD40 expression (Supplementary Fig. 2*C*). Flow cytometry analysis of vimentin$^+$ cells confirmed that rescue of CD40 occurred in Trg-CD40 but not in Trg-Ctr mice and that levels of CD40 expression in vimentin$^+$ cells were similar in B6 and Trg-CD40 mice (Supplementary Fig. 2*D*). Staining with an antibody against CRALBP, a specific Müller cell marker, confirmed that CD40$^+$ cells from Trg-CD40 mice were Müller cells (Supplementary Figs. 2*E* and 3*A*). The pattern of GFAP expression was the same in B6 and transgenic mice and was detected in astrocytic processes present in the ganglion cell layer (Supplementary Fig. 2*F*). These results are in agreement with evidence that the GFAP promoter drives gene expression in Müller cells independently of endogenous GFAP gene activity (24). Moreover, GFAP$^+$ astrocytes lacked detectable CD40 (Supplementary Fig. 2*G*) because expression driven by the GFAP transgene is lost in astrocytes from adult mice (24). Selective localization of CD40 in Trg-CD40 mice was further confirmed using immunohistochemistry and flow cytometry by the lack of detectable CD40 expression in the other retinal cells that express CD40 in wild-type mice: endothelial cells, microglia/macrophages, and ganglion cells, as well as lack of CD40 expression in leukocytes (Supplementary Figs. 2*G* and 3*B* and *C*). Thus, the transgenic system results in mice with CD40 rescue in Müller cells rather than astrocytes.

Müller Cell CD40 Promotes ICAM-1 Upregulation and Vascular Changes of Experimental Diabetic Retinopathy

Male B6, $CD40^{-/-}$, Trg-Ctr, and Trg-CD40 mice were rendered diabetic using STZ. Blood glucose concentrations, HbA_{1c} levels, and body weights of diabetic mice were similar ($P > 0.5$) (Supplementary Table 1). ICAM-1 expression is elevated in the diabetic retina (17) and promotes leukostasis (25,26). Compared with nondiabetic mice, ICAM-1 mRNA levels were increased in diabetic Trg-CD40 and B6 mice but not in diabetic Trg-Ctr or

CD40$^{-/-}$ mice (Fig. 1*A*). Diabetic B6 and Trg-CD40 mice showed increased ICAM-1 expression in retinal capillaries compared with nondiabetic controls (Fig. 1*B*). In contrast, diabetic CD40$^{-/-}$ and Trg-Ctr mice did not exhibit increased ICAM-1 expression (Fig. 1*B*).

Next, we determined whether diabetic Trg-CD40 mice develop increased retinal leukostasis. Diabetic Trg-CD40 and B6 mice showed a significant increase in the numbers of adherent leukocytes (Fig. 1*C*). Degenerate capillaries are a central feature of diabetic retinopathy. Diabetic B6 mice exhibited the expected increase in degenerate capillaries compared with nondiabetic animals, whereas diabetic mice lacking CD40 were protected from capillary degeneration (Fig. 1*D*). Diabetic Trg-CD40 animals showed increased degeneration of retinal capillaries similar to that seen in diabetic B6 mice (Fig. 1*D*). Thus, CD40 in Müller cells promotes ICAM-1 upregulation in the diabetic retina and vascular changes of early diabetic retinopathy.

CD40 Expressed in Müller Cells Promotes Upregulation of TNF-α, IL-1β, NOS2, and CCL2 mRNA Levels in the Diabetic Retina

TNF-α, IL-1β, NOS2, and likely CCL2 play a pathogenic role in diabetic retinopathy (3,5,6,27–30). We examined the effects of Müller cell CD40 on mRNA levels of these molecules. In contrast to diabetic Trg-Ctr mice, diabetic Trg-CD40 animals upregulated TNF-α, IL-1β, NOS2, and CCL2 mRNA levels (Fig. 2). Thus, expression of CD40 on Müller cells drives upregulation of TNF-α, IL-1β, NOS2, and CCL2 mRNA in the retina of diabetic mice.

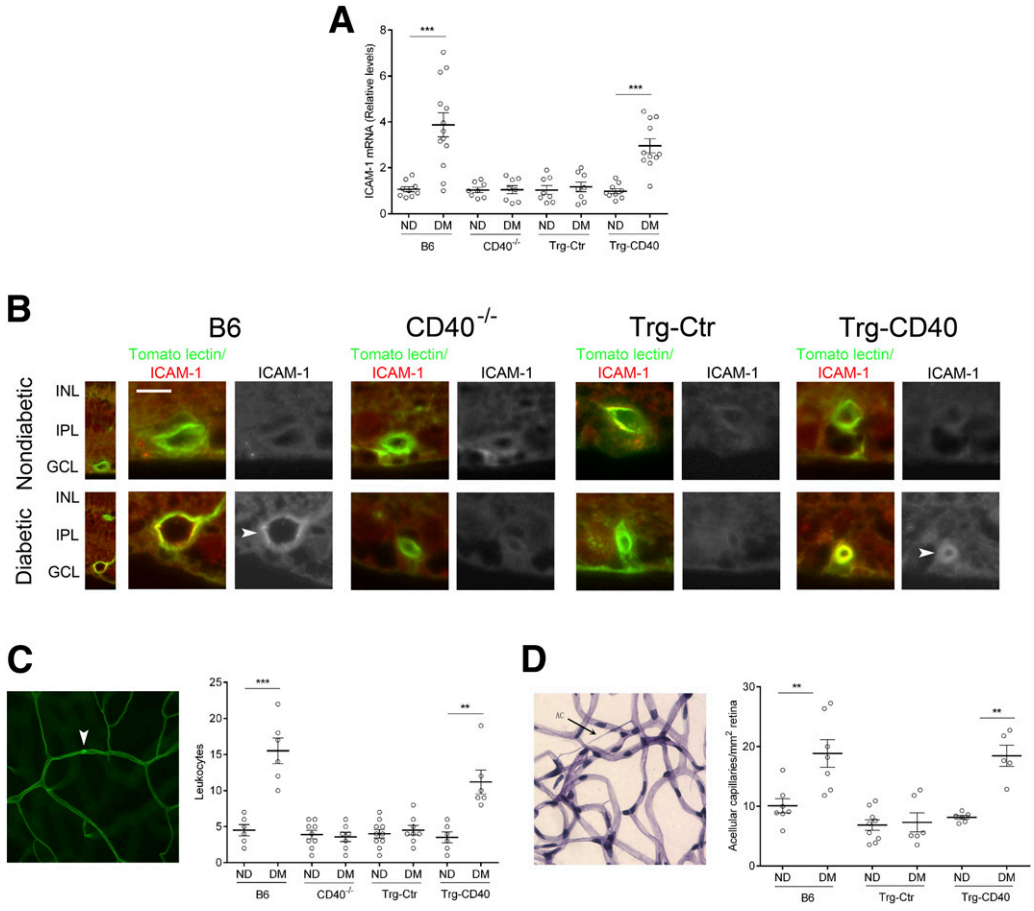

Figure 1—CD40 expression in Müller cells from diabetic mice promotes upregulation of ICAM-1 in the retina and vascular changes of early diabetic retinopathy. *A*: At 2 months of diabetes, retinas from diabetic B6, CD40$^{-/-}$, Trg-Ctr, and Trg-CD40 mice (DM) as well as from nondiabetic (ND) control animals were collected and used for mRNA extraction. mRNA levels of ICAM-1 were assessed by real-time quantitative PCR using 18S rRNA as the internal control. One nondiabetic B6 mouse was given an arbitrary value of 1, and data are expressed as the fold-increase compared with this animal. The horizontal bars represent mean ± SEM (n = 8–13 animals per group). *B*: At 2 months of diabetes, retinal sections were incubated with anti–ICAM-1 monoclonal antibody and tomato lectin (n = 6 mice/group). GCL, ganglion cell layer; INL, inner nuclear layer; IPL, inner plexiform layer. Arrowheads show blood vessels with increased ICAM-1 expression. Scale bar, 10 μm. *C*: At 2 months of diabetes, adherent leukocytes in the retinal vasculature of diabetic and nondiabetic control mice were quantified by labeling with concanavalin A. Representative image shows an adherent leukocyte (arrowhead) within the vasculature of a diabetic B6 mouse. *D*: At 8 months of diabetes, retinal digests were examined for the presence of degenerate capillaries. The horizontal bars represent the mean ± SEM (n = 6–10 animals per group). Representative image shows an acellular capillary (arrow) in the retinal digest of a diabetic B6 mouse. $^{**}P < 0.01$, $^{***}P < 0.001$ by ANOVA.

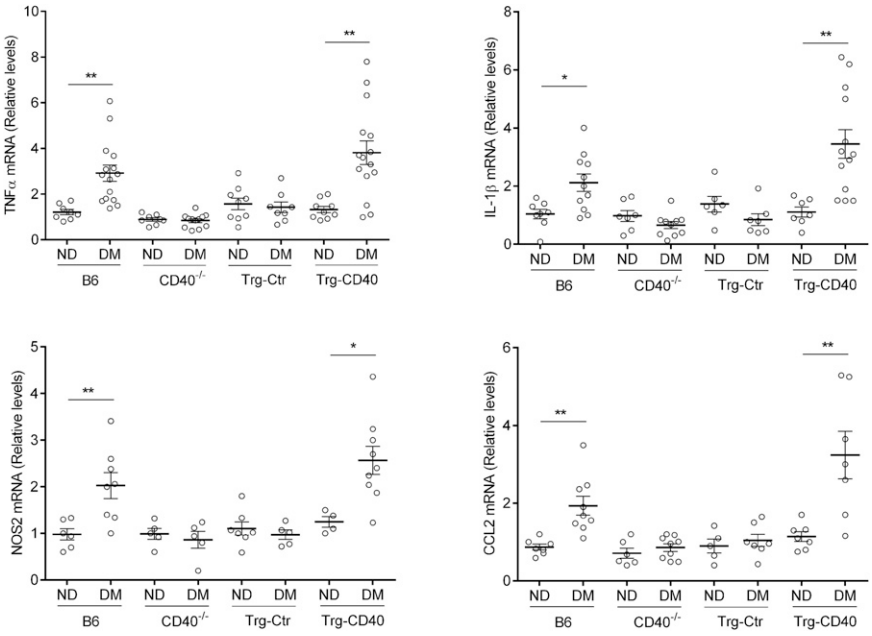

Figure 2—CD40 expression in Müller cells from diabetic mice (DM) restores upregulation of TNF-α, IL-1β, NOS2, and CCL2 mRNA in the retina. At 2 months of diabetes, retinas from diabetic B6, CD40$^{-/-}$, Trg-Ctr, and Trg-CD40 mice and from nondiabetic (ND) control animals were collected and used for mRNA extraction. mRNA levels were assessed by real-time quantitative PCR using 18S rRNA as the internal control. One nondiabetic B6 mouse was given an arbitrary value of 1, and data are expressed as the fold-increase compared with this animal. The horizontal bars represent the mean ± SEM (n = 7–15 animals per group). *P < 0.05, **P < 0.01 by ANOVA.

Müller Cells Do Not Secrete TNF-α or IL-1β in Response to CD40 Ligation

We examined the effects of CD40 stimulation on TNF-α and IL-1β production by Müller cells. CD40 expression in Müller cells is upregulated in diabetes (8). However, in agreement with other nonhematopoietic cells, primary Müller cells and Müller cell lines have very low levels of CD40 expression in vitro under basal conditions. To test whether CD40 ligation caused TNF-α and/or IL-1β secretion, we induced CD40 expression by transducing Müller cells with a CD40-encoding retroviral vector, an approach well suited to study the effects of CD40 signaling (9,11). CD40$^+$ primary human Müller cells or the CD40$^+$ human Müller cell line MOI-M1 failed to secrete TNF-α in response to CD154 (CD40 ligand) (Supplementary Fig. 4*A*). Although human Müller cells also failed to secrete IL-1β in response to CD154, a marked upregulation in CCL2 was detected (Supplementary Fig. 4*A*). We tested whether similar findings apply to Müller cells from rodents. The rat Müller cell line rMC-1, which expresses a chimera of the extracellular domain of human CD40, and the intracytoplasmic domain mouse CD40 (hmCD40) was incubated with human CD154. These cells upregulated ICAM-1 in response to human CD154, but TNF-α secretion was not detected (Supplementary Fig. 4*B*). Mouse Müller cells that express hmCD40 also failed to secrete TNF-α and IL-1β (Fig. 4*I*). Thus, CD40 stimulation of Müller cells does not increase TNF-α and IL-1β secretion.

Retinal Microglia/Macrophages but Not Müller Cells From Diabetic Trg-CD40 Mice Express TNF-α

We examined CCL2 and TNF-α expression by immunohistochemistry. CCL2 was detected in the end feet and stalks of Müller cells from diabetic Trg-CD40 mice but not in diabetic Trg-Ctr mice or nondiabetic animals (Fig. 3*A* and *B* and Supplementary Fig. 5*A*). In contrast, no TNF-α was detected in Müller cells from diabetic Trg-CD40 mice or mice from any other group (Fig. 3*C* and Supplementary Fig. 5*B*). Interestingly, TNF-α was observed in microglia/macrophages (Iba-1$^+$ cells) from diabetic Trg-CD40 mice (Fig. 3*D* and *E*). Analysis of multiple retinal sections revealed that 15% of microglia/macrophages in diabetic Trg-CD40 mice were TNF-α$^+$ (Fig. 3*D*). Thus, the presence of CD40 in Müller cells from diabetic mice drives CCL2 upregulation in Müller cells and upregulation of TNF-α in microglia/macrophages.

CD40-Activated Müller Cells Secrete ATP and Cause P2X$_7$ Receptor–Dependent Cytokine Production in Monocytes/Macrophages

We tested whether CD40-activated Müller cells induce IL-1β and TNF-α production in bystander monocytes/macrophages. We used monocytic cells that lack CD40 (MonoMac6) to avoid the effects of direct CD40 ligation on these cells. Similar to Müller cells, monocytic cells failed to secrete IL-1β and TNF-α in response to CD154 (Fig. 4*A*). In contrast, the addition of CD154 to coculture of CD40$^+$ human Müller cells with monocytic cells enhanced IL-1β and TNF-α production (Fig. 4*A*). Cytokine

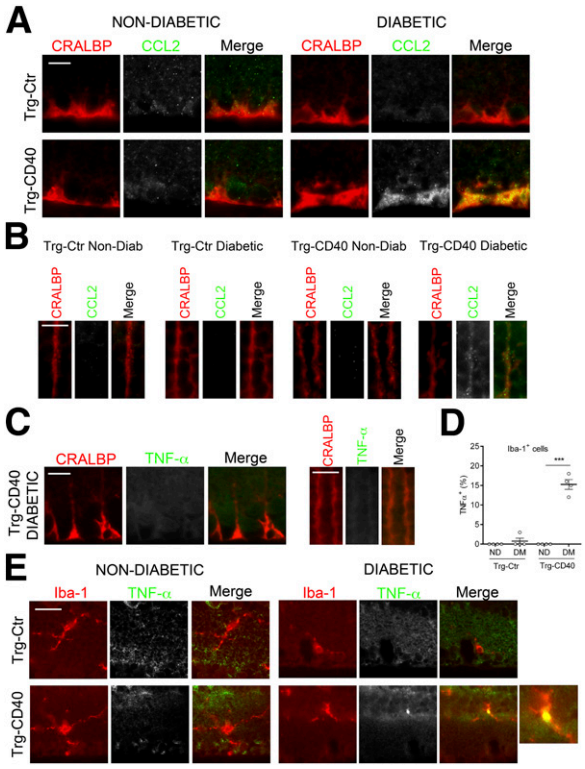

Figure 3—Diabetic transgenic mice that express CD40 in retinal Müller cells upregulate CCL2 in Müller cells and TNF-α in microglia/macrophages. *A* and *B*: Retinal sections from diabetic Trg-Ctr and Trg-CD40 mice at 2 months of diabetes and from nondiabetic controls were incubated with anti-CCL2 plus anti-CRALBP antibody. CCL2 expression at the level of Müller cell end feet (ganglion cell layer) (*A*) and at the level of Müller cells stalks (outer plexiform layer) (*B*). *C*: Sections were incubated with anti–TNF-α plus anti-CRALBP antibody. Müller cell end feet and stalk areas are shown. *D* and *E*: Retinal sections were incubated with anti–TNF-α monoclonal antibody plus anti–Iba-1 antibody. *D*: Sections were analyzed to determine the percentages of Iba-1$^+$ cells in diabetic mice (DM) and nondiabetic (ND) mice that were stained with anti–TNF-α antibody. ***$P < 0.001$. *E*: Images represent microglia/macrophages present in the inner plexiform layer ($n = 4$ mice/group). Scale bars, 10 μm.

production was driven by CD40 because IL-1β and TNF-α secretion were not significantly increased in cultures that contained Müller cells that were largely CD40$^-$ (Fig. 4*A*).

Secretion of ATP by astrocytes has been proposed to cause purinergic receptor–driven IL-1β production by microglia (31). To determine whether purinergic signaling mediates IL-1β and TNF-α production during the Müller cells/monocytic cells coculture, we tested whether CD40 ligation in Müller cells increases secretion of extracellular ATP. CD40$^+$ human Müller cells incubated with CD154 exhibited increased ATP release that was noted after 15 min of stimulation (Fig. 4*B* and *C*). Similar results were observed in mouse Müller cells (Fig. 4*D*).

Extracellular ATP binds purinergic receptors, among which P2X$_7$ is central to IL-1β secretion (32). The addition of a specific P2X$_7$ receptor inhibitor (A-438079) to CD40$^+$ human Müller cells and monocytic cells ablated the enhanced IL-1β production triggered by CD154 (Fig. 4*E*). It appeared unlikely that the P2X$_7$ receptor inhibitor acted on Müller cells because they did not exhibit detectable functional P2X$_7$ receptor (Supplementary Fig. 6*A*). To confirm that monocytes but not Müller cells were responsible for cytokine production, cells were incubated with a purinergic receptor ligand. 3′-*O*-(4-benzoyl)benzoyl (Bz)-ATP (100–300 μmol/L) enhanced cytokine production by monocytic cells but not by Müller cells (Supplementary Fig. 6*B*). In contrast to IL-1β, P2X$_7$ receptor inhibitor did not decrease TNF-α production triggered by addition of CD154 to CD40$^+$ human Müller cells cultured with monocytic cells (Fig. 4*F*). Similarly, knockdown of the P2X$_7$ receptor in monocytic cells impaired IL-1β production but not TNF-α when these cells were incubated with CD40$^+$ Müller cells plus CD154 (Fig. 4*G* and *H*). Of relevance, although P2X$_7$ receptor is central to IL-1β secretion, other P2 receptors in monocytic cell lines induce TNF-α production (33). Next, we examined the role of P2X$_7$ receptor in cytokine production by primary mouse macrophages. Whereas mouse hmCD40$^+$ Müller cells or mouse BMM failed to secrete IL-1β or TNF-α when incubated with human CD154, incubation of hmCD40$^+$ Müller cells plus BMM resulted in increased IL-1β and TNF-α production after human CD154 stimulation (Fig. 4*I*). No significant increase in cytokine production was noted when BMM from P2X$_7^{-/-}$ mice were used (Fig. 4*I*). Thus, CD40-activated Müller cells secrete extracellular ATP and induce P2X$_7$ receptor–mediated cytokine production in monocytes/macrophages.

CD40 Appears to Induce ATP Release in Müller Cells Through PLCγ1

ATP can be released after an increase in intracytoplasmic Ca^{2+} (34), and CD40 elevates intracytoplasmic Ca^{2+} levels (35,36). We examined the effects of BAPTA-AM (chelator of intracellular Ca^{2+}) on ATP release. Incubation with BAPTA-AM impaired CD40-mediated ATP release in Müller cells (Fig. 5*A*). Next, we examined whether CD40 ligation in Müller cells causes phosphorylation of PLCγ1, a signaling molecule that increases intracytoplasmic Ca^{2+} (37). CD40 ligation caused a rapid PLCγ1 phosphorylation at Tyr783, a marker of PLCγ1 activation (Fig. 5*B*). ATP release was impaired when Müller cells were treated with the PLC inhibitor U73122 but not when incubated with the inactive analog U73343 (Fig. 5*C*). Thus, CD40 ligation phosphorylates PLCγ1 and appears to cause PLCγ1-dependent ATP release in Müller cells.

Deficiency of P2X$_7$ Receptor or Administration of a P2X$_7$ Receptor Inhibitor Impairs Upregulation of TNF-α, IL-1β, ICAM-1, and NOS2 in the Retina of Diabetic Mice

We examined P2X$_7$ receptor expression to explore its in vivo role in the diabetic retina. P2X$_7$ receptor mRNA levels were enhanced in the retinas of diabetic Trg-CD40 mice (Fig. 6*A*). Immunohistochemistry indicated that microglia/macrophages from diabetic Trg-CD40 mice exhibited increased P2X$_7$ receptor expression (Fig. 6*B*

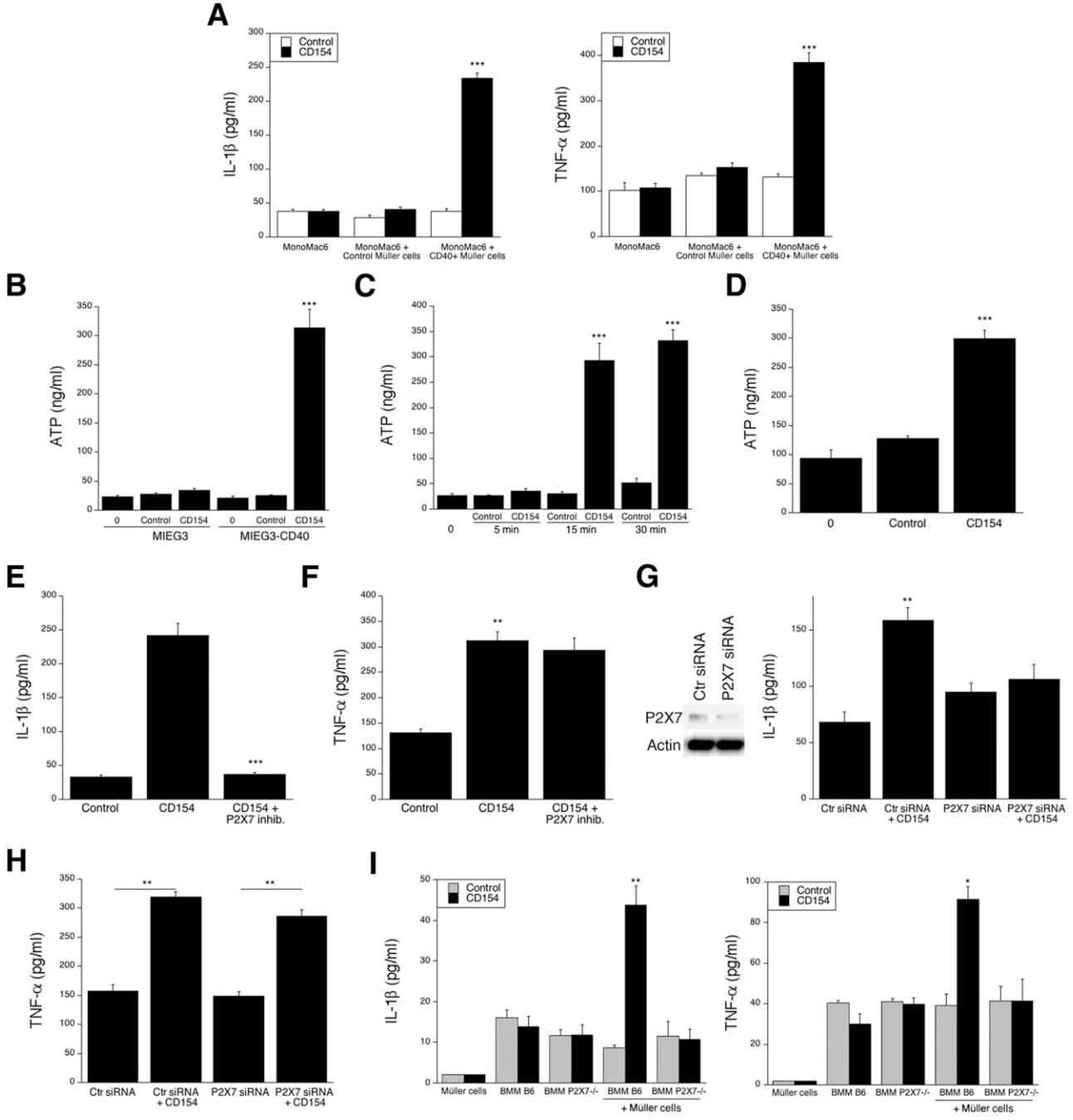

Figure 4—CD40-activated Müller cells secrete ATP and induce $P2X_7$ receptor–dependent production of cytokines by monocytic cells and macrophages. *A*: Control human Müller cell (transduced with empty retroviral vector MIEG3) and $CD40^+$ human Müller cells (transduced with CD40-encoding retroviral vector MIEG3-CD40) were incubated with $CD40^-$ human monocytic cell lines (MonoMac6) with or without CD154. Cytokines were measured in supernatants by ELISA at predetermined optimal time points (24 h for IL-1β and 4 h for TNF-α). *B*: Human Müller cell line was incubated with or without CD154. Concentrations of extracellular ATP at time 0 and at 15 min of incubation are shown. *C*: $CD40^+$ Müller cells were incubated with CD154 and concentrations of extracellular ATP were measured at different times. *D*: Mouse Müller cells transduced with the hmCD40-encoding retroviral vector were incubated with or without human CD154. $CD40^+$ human Müller cells were incubated with MonoMac6 cells with or without CD154 in the presence or absence of the $P2X_7$ receptor inhibitor A-438079, and IL-1β (*E*) and TNF-α (*F*) were measured in supernatants by ELISA. $CD40^+$ human Müller cells were incubated with MonoMac6 cells transfected with control or $P2X_7$ receptor siRNA, and IL-1β (*G*) and TNF-α (*H*) were measured in supernatants by ELISA. *I*: Mouse Müller cells that express hmCD40 were incubated with BMM from B6 or $P2X_7^{-/-}$ mice, and IL-1β (*I*) and TNF-α (*J*) were measured by ELISA. Results are presented as mean ± SEM ($n = 3$). $^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ by Student *t* test.

and *C*). Next, we examined the role of $P2X_7$ receptor on TNF-α and IL-1β expression in diabetic mice and also examined ICAM-1 and NOS2 because retinal ICAM-1 upregulation in diabetes is TNF-α dependent (38) and TNF-α and IL-1β promote NOS2 expression (39). Compared with B6 mice, diabetic $P2X_7^{-/-}$ mice exhibited

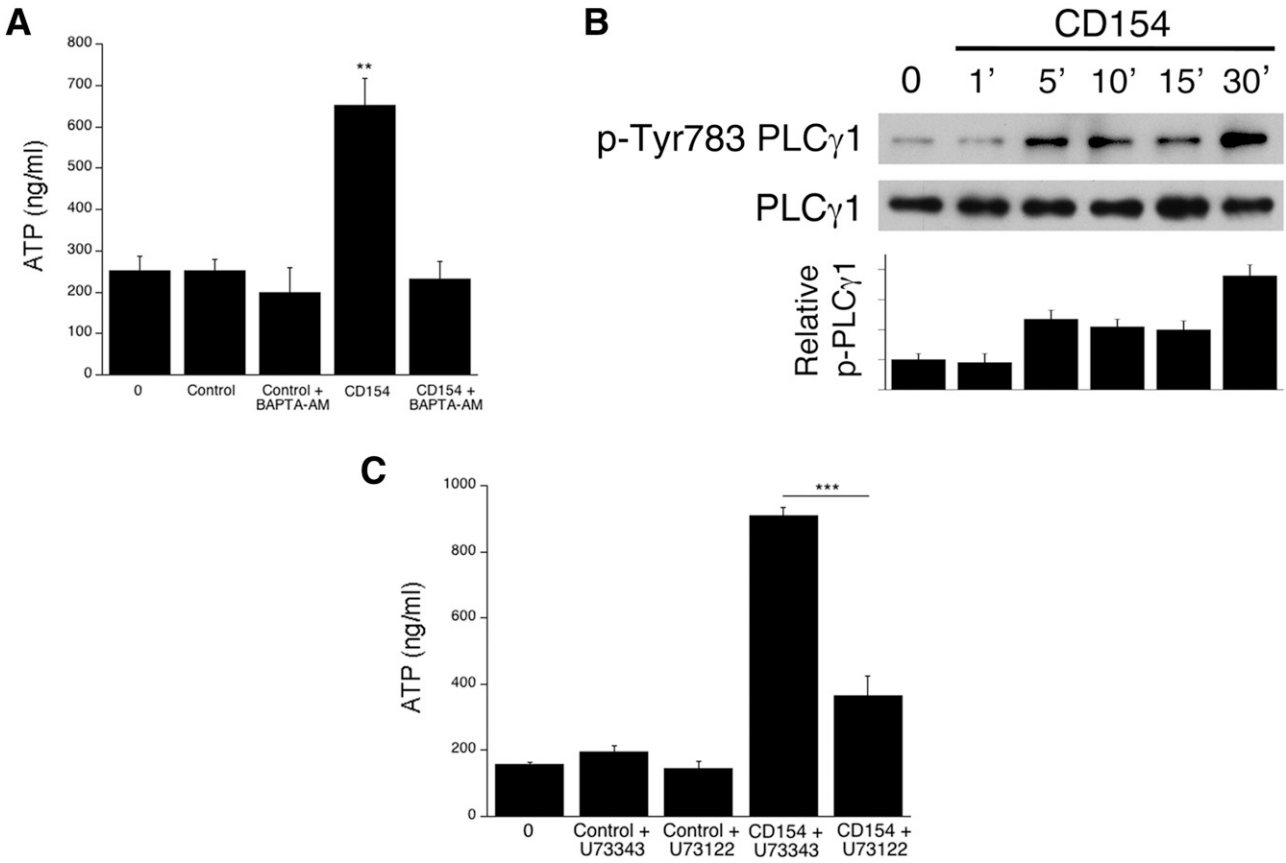

Figure 5—CD40 ligation in Müller cells causes Tyr783 phosphorylation (p) of PLCγ1 and PLC-dependent secretion of ATP. *A*: CD40$^+$ Müller cells were incubated with or without BAPTA-AM, followed by stimulation with CD154 and measurement of extracellular ATP. Concentrations of extracellular ATP at time 0 and at 15 min of incubation are shown. *B*: CD40$^+$ human Müller cells were incubated with CD154. Expression of p-Tyr783 PLCγ1 and total PLCγ1 were assessed by immunoblot. The bars represent quantification of relative p-Tyr783 PLCγ1 from three different experiments. *C*: CD40$^+$ Müller cells were incubated with U73122 or U73343, followed by stimulation with CD154 and measurement of extracellular ATP. Concentrations of extracellular ATP at time 0 and at 15 min of incubation are shown. Results are presented as mean ± SEM (n = 3). $^{**}P < 0.01$, $^{***}P < 0.001$ by Student t test.

impaired upregulation of TNF-α, IL-1β, ICAM-1, and NOS2 (Fig. 6*D*). We treated diabetic Trg-CD40 mice with the P2X$_7$ receptor inhibitor Brilliant Blue G (BBG). BBG did not affect blood glucose or HbA$_{1c}$ levels (data not shown) but did diminish diabetes-induced TNF-α, IL-1β, ICAM-1, and NOS2 upregulation (Fig. 6*E*). Together, the presence of CD40 in Müller cells from diabetic mice drives upregulation of retinal TNF-α, IL-1β, ICAM-1, and NOS2 through a P2X$_7$ receptor–dependent mechanism.

DISCUSSION

We report that expression of CD40 in Müller cells is sufficient for upregulation of inflammatory molecules in the diabetic retina and development of early diabetic retinopathy. In diabetes, CD40 on Müller cells drives upregulation of CCL2 in these cells; however, CD40 does not increase TNF-α and IL-1β secretion by Müller cells. CD40 stimulation in Müller cells causes release of extracellular ATP, an effect that appears dependent on PLCγ1. In turn, ATP released by CD40-activated Müller cells induces P2X$_7$ receptor–mediated secretion of TNF-α and IL-1β by macrophages. These effects are relevant in vivo because TNF-α is upregulated in microglia/macrophages from diabetic mice that express CD40 in Müller cells and mice treated with BBG are protected from diabetes-induced upregulation of TNF-α and IL-1β and also upregulation of ICAM-1 and NOS2. The role of P2X$_7$ is not only applicable to transgenic mice because diabetic P2X$_7^{-/-}$ mice also exhibit diminished inflammatory molecule upregulation. These findings support a model of amplification of inflammation whereby CD40 engagement in Müller cells triggers an inflammatory response not only in these cells but also in bystander myeloid cells in a manner dependent on the ATP-P2X$_7$ receptor pathway.

Extracellular ATP acts not only as a neurotransmitter but also as a messenger that triggers cytokine production by macrophages/microglia. We uncovered that CD40 induced the release of extracellular ATP. CD40 stimulation caused rapid Tyr783 phosphorylation of PLCγ1 in Müller cells, and PLC inhibition prevented release of extracellular ATP after CD40 ligation. Indeed, PLC mediates ATP release (40). Work presented here strongly supports

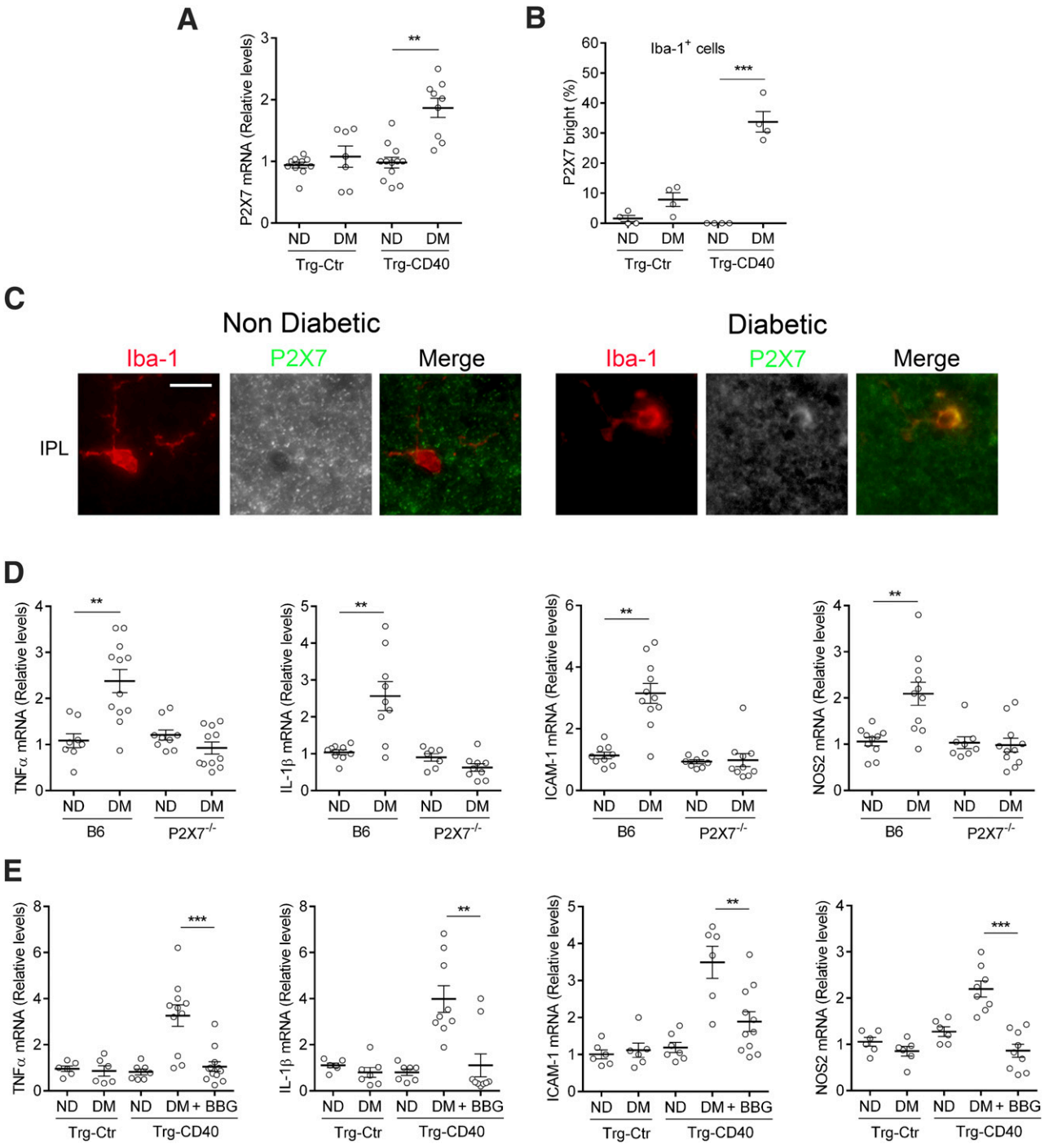

Figure 6—Diabetic transgenic mice that express CD40 in retinal Müller cells upregulate $P2X_7$ receptor in microglia/macrophages, and administration of the $P2X_7$ receptor inhibitor BBG impairs upregulation of retinal mRNA levels of TNF-α and IL-1β. *A*: At 2 months of diabetes, retinas from diabetic (DM) and nondiabetic (ND) Trg-Ctr and Trg-CD40 mice were collected and used for mRNA extraction. mRNA levels of $P2X_7$ receptor were assessed by real-time quantitative PCR using 18S rRNA as the internal control. One nondiabetic Trg-Ctr mouse was given an arbitrary value of 1, and data are expressed as the fold-increase compared with this animal. The horizontal bars represent the mean ± SEM (n = 7–11 animals per group). *B* and *C*: Sections from diabetic and nondiabetic Trg-CD40 mice were incubated with anti-$P2X_7$ receptor plus anti–Iba-1 antibodies. *B*: Sections were analyzed to determine the percentages of Iba-1^+ cells that stained brightly with anti-$P2X_7$ receptor anbitody. *C*: Images represent microglia/macrophages present in the inner plexiform layer (IPL) (n = 4 mice/group). Scale bar, 10 μm. *D*: At 2 months of diabetes, retinas from diabetic and nondiabetic B6 and $P2X_7^{-/-}$ mice were collected and used to measure TNF-α, IL-1β, ICAM-1, and NOS2 mRNA levels. One nondiabetic B6 mouse was given an arbitrary value of 1, and data are expressed as the fold-increase compared with this animal (n = 7–12 animals per group). *E*: Diabetic Trg-CD40 mice were treated with BBG or vehicle daily for 28 days beginning at 1 month of diabetes. Retinas from these animals and from diabetic and nondiabetic Trg-Ctr were collected and used to measure TNF-α, IL-1β, ICAM-1, and NOS2 mRNA levels (n = 6–11 mice per group.) $^{**}P < 0.01$, $^{***}P < 0.001$ by ANOVA.

that purinergic signaling promotes proinflammatory cytokine expression in microglia/macrophages because *1*) administration of the $P2X_7$ inhibitor BBG impaired upregulation of TNF-α and IL-1β in diabetic mice that express CD40 restricted to Müller cells, and *2*) diabetic $P2X_7^{-/-}$ mice were protected from upregulation of these cytokines. These findings identified a mechanism that enables CD40 expressed in nonhematopoietic cells to induce macrophages/microglia to produce TNF-α and IL-1β, cytokines pivotal in inflammation. This mechanism would circumvent the inability or poor capacity of CD40 to trigger secretion of these cytokines in nonhematopoietic cells.

The level of $P2X_7$ receptor expression is functionally relevant because increased receptor expression is sufficient to cause microglia activation even in the absence of pathologic stimuli (41). Thus, the importance of the $P2X_7$ receptor in diabetic retinopathy is supported not only by the studies performed in $P2X_7^{-/-}$ mice and in mice treated with BBG but also by the upregulation of the $P2X_7$ receptor noted in retinal microglia/macrophages. Moreover, $P2X_7$ receptor upregulation also occurs in diabetic B6 mice (J.-A.C.P., C.S.S., unpublished data). $P2X_7$ receptor is key for IL-1β and TNF-α secretion by microglia/macrophages (42–45). Both cytokines contribute to diabetes-induced degeneration of retinal capillaries (5,6). The observation that the concentration of Bz-ATP (100–300 μmol/L) that induced IL-1β and TNF-α production in monocytic cells appeared to be higher than the ATP concentration detected in ATP assays does not detract from the role of purinergic signaling in IL-1β and TNF-α upregulation. The assay based on firefly luciferase underestimates ATP concentrations in the intercellular space. Assays based on targeting luciferase to the surface of intact cells revealed higher concentrations of ATP at the cell surface (100–200 μmol/L) (46). In addition, $P2X_7$ receptor upregulation in retinal microglia/macrophages may increase their sensitivity to ATP (47).

Serum CD154 levels are elevated in diabetic mice, and soluble CD154 is biologically active (48). Thus, these results together with the prior demonstration of increased CD40 expression in retinal cells indicate that the CD40-CD154 pathway is likely activated in diabetes. Of relevance, plasma CD15 is increased in patients with diabetic retinopathy (49). Retinal CD154 levels are also likely increased because microthrombosis occurs in diabetic retinopathy and activated platelets express CD154 (50). Differences in soluble CD154 concentrations may contribute to the susceptibility to experimental diabetic retinopathy. However, we have not detected differences in serum CD154 concentrations between diabetic B6 and $P2X_7^{-/-}$ mice, it is unlikely that CD40 expression in Müller cells affects circulating CD154, and CD40 upregulation is sufficient to markedly increase proinflammatory responses upon CD40 ligation. Although we did not detect CD40 expression in cells other than Müller glia, we cannot rule out that potential ectopic CD40 expression could contribute to the results observed. Finally, although these studies revealed the importance of CD40 in Müller cells for the pathogenesis of experimental diabetic retinopathy, CD40 signaling at the level of microglia/macrophages and/or endothelial cells likely also participates in the development of this disease.

In summary, this study uncovered the pivotal role CD40 expressed in a nonhematopoietic compartment and the purinergic-mediated recruitment of proinflammatory responses in microglia/macrophages in the development of inflammatory responses. These findings may also be relevant to other diseases driven by CD40, including inflammatory bowel disease, atherosclerosis, and lupus nephritis. CD40 expressed in nonhematopoietic cells in intestine, blood vessels, and kidney may trigger the release of extracellular ATP and engagement of purinergic receptors present in infiltrating myeloid cells. Finally, these results may have therapeutic implications. Inhibition of CD40 can lead to a therapeutic approach against inflammatory disorders. Signaling pathways downstream of CD40 have different relative roles in inducing proinflammatory responses in hematopoietic and nonhematopoietic cells (11). Targeting a signaling pathway in nonhematopoietic cells may inhibit proinflammatory responses in these cells and in neighboring myeloid cells.

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