CD40 Expressed in Endothelial Cells Promotes Upregulation of ICAM-1 But Not Pro-Inflammatory Cytokines, NOS2 and P2X$_7$ in the Diabetic Retina

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Purpose. CD40 is an upstream inducer of inflammation in the diabetic retina. CD40 is upregulated in retinal endothelial cells in diabetes. The purpose of this study was to determine whether expression of CD40 in endothelial cells is sufficient to promote inflammatory responses in the retina of diabetic mice.

Methods. Transgenic mice with CD40 expression restricted to endothelial cells (Trg-CD40 EC), transgenic control mice (Trg-Ctr), B6, and CD40$^{-/-}$ mice were made diabetic using streptozotocin. Leukostasis was assessed using FITC-conjugated ConA. Pro-inflammatory molecule expression was examined by real-time PCR, immunohistochemistry, ELISA, or flow cytometry. Release of ATP was assessed by ATP bioluminescence.

Results. Diabetic B6 and Trg-CD40 EC mice exhibited increased retinal mRNA levels of ICAM-1, higher ICAM-1 expression in endothelial cells, and increased leukostasis. These responses were not detected in diabetic mice that lacked CD40 (CD40$^{-/-}$ and Trg-Ctr). Diabetic B6 but not Trg-CD40 EC mice upregulated TNF-α, IL-1β, and NOS2 mRNA levels. CD40 stimulation in retinal endothelial cells upregulated ICAM-1 but not TNF-α, IL-1β, or NOS2. CD40 ligation did not trigger ATP release by retinal endothelial cells or pro-inflammatory cytokine production in bystander myeloid cells. In contrast to diabetic B6 mice, diabetic Trg-CD40 EC mice did not upregulate P2X$_7$ mRNA levels in the retina.

Conclusions. Endothelial cell CD40 promotes ICAM-1 upregulation and leukostasis. In contrast, endothelial cell CD40 does not lead to pro-inflammatory cytokine and NOS2 upregulation likely because it does not activate purinergic-mediated pro-inflammatory molecule expression by myeloid cells or induce expression of these pro-inflammatory molecules in endothelial cells.

Keywords: endothelial cells, inflammation, TNF, IL-1, ATP

Diabetic retinopathy is a leading cause of visual impairment in the world.[1] Although multiple factors likely contribute to the pathogenesis of diabetic retinopathy,[2,3] chronic low-level inflammation plays an important role in the development and progression of this disease.[4–6] CD40 has been identified as a central upstream regulator of various inflammatory responses in the diabetic retina and a key component for the development of experimental diabetic retinopathy.[7–10] CD40 is a member of the TNF receptor superfamily that is normally expressed at low levels in endothelial cells, Müller cells, microglia/macrophages ganglion cells, and retinal pigment epithelial cells in the retina.[11] Its expression in endothelial cells, Müller cells, and microglia/macrophages is increased in the diabetic retina.[8] In vitro studies revealed that CD40 ligation induces important pro-inflammatory responses that include upregulation of ICAM-1 as well as increased production of CCL2 and CXCL1 in retinal endothelial cells (the latter in the presence of IL-1β)[7,8,12,13]; increased production of CCL2, nitric oxide, PGE$_2$, and upregulation of NOS2 and ICAM-1 in Müller cells[7–9]; increased production of TNF-α, IL-1β, and CCL2 by myeloid cells.[14,15] Importantly, diabetic CD40$^{-/-}$ mice are protected from upregulation of ICAM-1, TNF-α, IL-1β, NOS2, leukostasis, and the development of experimental diabetic retinopathy.[7–9]

Studies using diabetic transgenic mice revealed that expression of CD40 restricted to Müller cells is sufficient for upregulation of ICAM-1, TNF-α, IL-1β, CCL2, and NOS2 mRNA levels as well as for development of leukostasis and capillary degeneration.[9] The presence of CD40 in Müller cells of the diabetic retina leads to upregulation of CCL2 in these cells.[9] Interestingly, Müller cell CD40 also induces pro-inflammatory cytokine production in bystander microglia/macrophages in diabetic mice.[9] CD40 ligation in Müller cells triggers release of extracellular ATP that engages the purinergic receptor P2X$_7$ in macrophages resulting in P2X$_7$-dependent secretion of TNF-α and IL-1β by these cells.[9] Production of these pro-inflammatory cytokines likely amplifies ICAM-1 upregulation in endothelial cells and NOS2 upregulation in the diabetic retina.[9] Those studies

uncovered the CD40-ATP-P2X$_7$ pathway as a mechanism by which CD40 expressed in nonhematopoietic cells recruits inflammatory responses in myeloid cells.

Although the work described above demonstrated that CD40 in Müller cells is a key driver of inflammation and development of early experimental diabetic retinopathy, endothelial cells are also deemed to be important players in the pathogenesis of diabetic retinopathy. Given that in vivo upregulation of CD40 is a feature of CD40-driven inflammatory disorders, the fact that retinal endothelial cells upregulate CD40 in diabetes suggests that endothelial cell CD40 may contribute to inflammation in the diabetic retina. Using transgenic mice with expression of CD40 targeted to endothelial cells, we report that indeed endothelial cell CD40 promotes upregulation of ICAM-1 in these cells as well as leukostasis. However, expression of CD40 in endothelial cells does not lead to upregulation of TNF-α, IL-1β, and NOS2 in the diabetic retina likely because retinal endothelial cells not only do not express appreciable amounts of the molecules upon CD40 ligation, but also do not secrete extracellular ATP in response to CD40 stimulation and are unable to recruit pro-inflammatory responses in bystander myeloid cells.

MATERIALS AND METHODS

Mice

A binary tetracycline (Tet)-repressible transgenic mouse system that results in rescue of CD40 in endothelial cells has been previously reported.[16] The driver mice consisted of well-characterized heterozygous animals that express the Tet-repressible transactivator (tTA) under the control of the endothelial cell promoter *Tie1* (Tie1-tTA mice).[16] The responder mice were homozygous animals containing mouse CD40 cloned downstream of the Tet operator sequence (Tet^{OS}) promoter.[16] Mice are on a CD40$^{-/-}$ (B6) background.[16] Both lines were bred and offspring were genotyped by PCR of genomic DNA.[16] Double-transgenic offspring exhibit CD40 rescue in endothelial cells, whereas single-transgenic offspring lack CD40 rescue.[16] B6 and CD40$^{-/-}$ mice were originally purchased from Jackson Laboratories (Bar Harbor, ME, USA). All mice were bred at the Animal Resource Center (Case Western Reserve University). Male mice (12–16 weeks) were used for this study. Mice were randomly assigned to experimental groups. This study was carried out in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. The authors adhered to the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. The protocol was approved by the Institutional Animal Care and Use Committee of Case Western Reserve University School of Medicine (protocol number 2015-0130).

Induction of Diabetes

Mice were made diabetic using streptozotocin (STZ) as described.[8,9] Fasted mice (20–25 gram [g] body weight) received intraperitoneal injections of STZ (60 mg/kg; MP Biomedicals, Solon, OH, USA) daily for 5 days. Blood glucose was monitored and mice were considered diabetic when it reached 250 mg/mL. Glycated hemoglobin was measured at 2 months (Crystal Chem, Elk Grove Village, IL, USA). Mice received insulin based on weekly weight to prevent weight loss while maintaining chronic hyperglycemia (target range 350–550 mg/mL). Insulin dosing was determined for each animal individually (0–0.2 units of NPH insulin subcutaneously, 0–3 times per week). The insulin requirement was similar for all groups of diabetic mice.

Leukostasis

The number of leukocytes adherent to the retinal vasculature was assessed at 2 months of diabetes as described.[8,9] Briefly, fluorescent-coupled concanavalin A lectin (20% g/mL; Vector Laboratories, Burlingame, CA, USA) was infused into mouse eyes via perfusion with PBS. Leukostasis was evaluated by counting fluorescent leukocytes in retinal flat mounts using fluorescence microscopy.

Immunohistochemistry

Paraffin-embedded eyes were treated with proteinase K or citrate buffer. Sections were incubated with antibodies against CD40 (BioLegend, San Diego, CA, USA), ICAM-1 (eBiosciences, San Diego, CA, USA), Iba-1 (Wako Chemicals, Richmond, VA, USA), or TNF-α (Abcam, Cambridge, UK, USA). Fluorescent secondary antibodies were from Jackson ImmunoResearch Laboratories (West Grove, PA, USA). Sections incubated without primary antibodies were also included. Sections were also labeled with tomato lectin-DyLight 488 (Vector Laboratories, Burlingame, CA, USA). Tubular tomato lectin$^+$ structures represent endothelial cells. Slides were analyzed using an Olympus FV1200 IX-83 confocal microscope equipped with an UPlanSApa 60x objective, N.A. 1.35 oil. Images were acquired using FLUOVIEW version 4.2b. Images were processed in Photoshop CC 19.1.1. using similar linear adjustments for all samples.

Real-Time Quantitative PCR

RNA was isolated with RNeasy kit (QIAGEN, Hilden, Germany) and reverse transcribed to cDNA with SuperScript III reverse transcriptase (QIAGEN). The cDNA was used as template for real time (RT)-PCR using SYBER green PCR Master Mix with primers for ICAM-1, CCL2, TNF-α, IL-1β, IL-6, CXCL1, NOS2, and P2X$_7$.[9] Gene expression was assessed using a StepOne Real Time PCR system (ABI 7900 Sequence Detection System). The cDNA samples were run in triplicate. Samples were normalized according to the content of 18S rRNA.

Cells

Primary human retinal endothelial cells (Cell Systems, Kirkland, WA, USA) and the Müller cell line MIO M1 (gift from Dr. Gloria Limb, University College London, London, UK; >95% vimentin$^+$, cellular retinaldehyde binding protein [CRALBP]$^+$, and GFAP$^-$) were transduced with retroviruses containing MIEG3 backbone (empty) or MIEG3-CD40 expression vectors as described.[15] In certain experiments, endothelial cells were cultured in low glucose medium (5.5 mM) or high glucose medium (25 mM). Cells were treated with multimeric human CD154 (CD40 ligand; gift from Dr. Richard Kornbluth, Multimeric Biotherapeutics Inc., La Jolla, CA, USA).[12] Incubation with a nonfunctional CD154 mutant (T147N) was used as control. The human monocytic cell line Monomac6 cells (gift from Rene de Waal Malefyt, DNAX Research Institute, Palo Alto, CA, USA)

TABLE. Average Weight, Blood Glucose, and HbA$_{1c}$ in Control and Diabetic Mice

Strain	Group	Duration (mo)	n	Weight (g)	Glucose (mg/mL)	HbA$_{1c}$ (%)
B6	Control	2	7	30 ± 1.8	147 ± 7.1	3.3 ± 0.2
	Diabetic	2	9	25.4 ± 1.3	500 ± 14	9.7 ± 0.5
CD40$^{-/-}$	Control	2	7	29 ± 1.6	148 ± 7.7	3.4 ± 0.1
	Diabetic	2	9	25.8 ± 0.7	528 ± 18	10.3 ± 0.4
Trg-Ctr	Control	2	7	29.5 ± 1.6	149.4 ± 8	3.4 ± 0.2
	Diabetic	2	9	24.4 ± 0.5	538 ± 9	9.3 ± 0.8
Trg-CD40 EC	Control	2	7	28.5 ± 1.4	151 ± 9.1	3.4 ± 0.2
	Diabetic	2	9	24.6 ± 0.8	554 ± 9	9.5 ± 0.7

pretreated with IFN-γ (100 IU/mL; PeproTech, Rocky Hill, NJ, USA) with or without LPS (100 ng/mL; Sigma Aldrich, St. Louis, MO, USA; to promote IL-1β secretion) were co-incubated with retinal endothelial cells.

Measurement of Extracellular ATP

The ecto-ATPase inhibitor β,γ-methylene–ATP (300 mmol/L; Sigma-Aldrich) was added before incubation with CD154. Media was collected at 0, 5, 15, 30, 60, and 120 minutes. Extracellular ATP was quantified using an ATP bioluminescence assay kit and an ATP standard curve (Sigma Aldrich) (23). A Turner Designs (TD 20/20) luminometer was used to quantify luminescence.[17] ATP concentrations were calculated using an ATP standard curve.

ELISA

Commercial ELISA kits were used to measure concentrations of TNF-α, IL-1β (eBiosciences, San Diego, CA, USA) and CCL2 (R&D Systems, Minneapolis, MN, USA) in tissue culture supernatants. The lowest limits of detection for the assays were 7.8, 0.5, and 16 pg/mL, respectively.

Statistical Analysis

Results are expressed as the mean ± standard error of the mean (SEM). Data were analyzed by two-tailed Student's *t*-test or ANOVA. Differences were considered statistically significant at $P < 0.05$.

RESULTS

CD40 Expressed in Endothelial Cells Promotes ICAM-1 Upregulation and Leukostasis in Diabetic Mice

We utilized previously described CD40$^{-/-}$ transgenic mice where CD40 expression is rescued in endothelial cells[16] in order to examine the role of endothelial cell CD40 in regulation of inflammatory responses in the diabetic retina. The mice are based on a binary Tet repressible system. The driver line consisted of CD40$^{-/-}$ mice (B6 background) that are heterozygous for expression of the Tet-repressible tTA under the control of the endothelial cell promoter *Tie1* (Tie1-tTA mice)[16,18,19] (Fig. 1A). The responder line consisted of homozygous CD40$^{-/-}$ mice containing mouse CD40 cloned downstream of the Tet^{OS} promoter.[9,16] Mating Tie1-tTA mice with Tet^{OS} CD40 animals results in either double transgenic offspring (Trg-CD40 EC) shown to exhibit rescued CD40 expression in endothelial cells, or single transgenic mice (Trg-Ctr; carrying only Tet^{OS} CD40) shown to lack rescue of

CD40.[16] As previously reported,[16] compared to Trg-Ctr mice, Trg-EC CD40 mice exhibit rescue of CD40 expression in retinal endothelial cells (see Fig. 1A). Similar results have been reported in endothelial cells from other tissues (choroid, brain, lungs, and spleen).[16] Moreover, microglia and other leukocyte subsets in Trg-EC CD40 mice are known to remain CD40$^-$.[16] Finally, the levels of CD40 in endothelial cells from Trg-CD40 EC mice are reported to be similar to those of B6 mice.[16]

Male B6, CD40$^{-/-}$, Trg-Ctr, and Trg-EC CD40 mice were made diabetic using STZ. Blood glucose, HbA$_{1c}$ levels, and body weights of diabetic mice were similar in all groups ($P > 0.5$; Table). ICAM-1 expression is elevated in retinal endothelial cells from patients with diabetes and rodents promoting leukostasis.[20–22] Indeed, diabetic B6 mice exhibited increased ICAM-1 mRNA levels compared to non-diabetic animals (Fig. 1B). Diabetic mice lacking CD40 (CD40$^{-/-}$ and Trg-Ctr) did not exhibit ICAM-1 upregulation. In contrast, ICAM-1 mRNA levels were significantly increased in diabetic Trg-CD40 EC mice, although these levels appeared lower than those observed in diabetic B6 mice (see Fig. 1B). We used immunohistochemistry to examine ICAM-1 expression in retinal endothelial cells. Diabetic B6 and Trg-CD40 EC mice showed increased ICAM-1 expression in retinal capillaries compared to non-diabetic controls (Fig. 1C). In contrast, diabetic CD40$^{-/-}$ and Trg-Ctr mice did not exhibit increased ICAM-1 expression (see Fig. 1C). Next, we determined whether diabetic Trg-CD40 EC mice develop increased retinal leukostasis. Not only diabetic B6 mice but also diabetic Trg-CD40 EC mice showed a significant increase in the numbers of adherent leukocytes, although leukostasis was somewhat lower in Trg-CD40 EC mice compared to B6 animals (Fig. 1D). Thus, expression of CD40 restricted to retinal endothelial cells is sufficient to promote ICAM-1 upregulation in these cells and leukostasis in the retinas of diabetic mice.
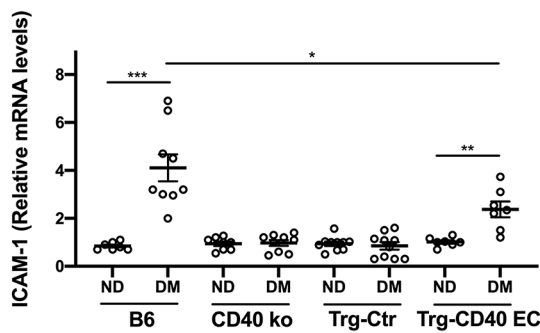
CD40 Expressed in Endothelial Cells Does Not Significantly Increase TNF-α, IL-1β, and NOS2 mRNA Levels in the Diabetic Retina

The expression of TNF-α, IL-1β, and NOS2 is increased in the diabetic retina[23–27] and these events are dependent on the presence of CD40.[9] In agreement with these studies, mRNA levels of TNF-α and IL-1β were increased in the retinas of diabetic B6 mice but not in CD40$^{-/-}$ animals (Figs. 2A, 2B). However, in contrast to ICAM-1, rescue of CD40 expression in endothelial cells failed to upregulate TNF-α and IL-1β levels in diabetic animals (see Figs. 2A, 2B). Moreover, whereas TNF-α was detected in retinal microglia/macrophages from diabetic B6 mice, no TNF-α was observed in diabetic Trg-CD40 EC mice (Fig. 2C). In
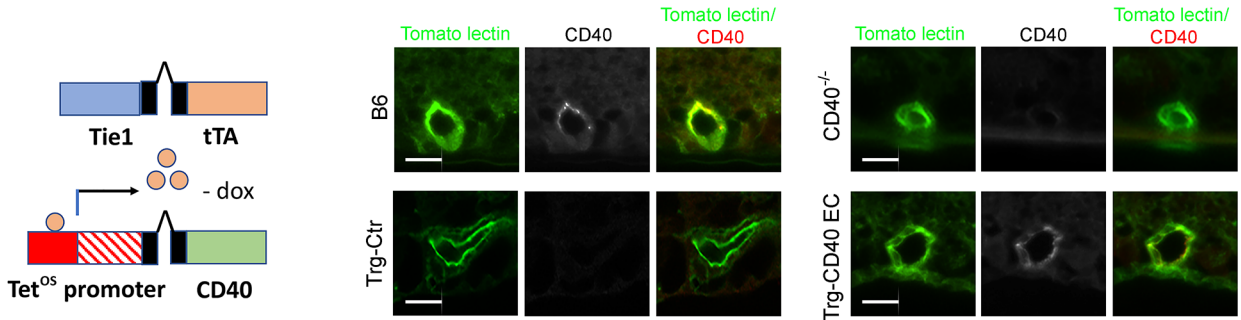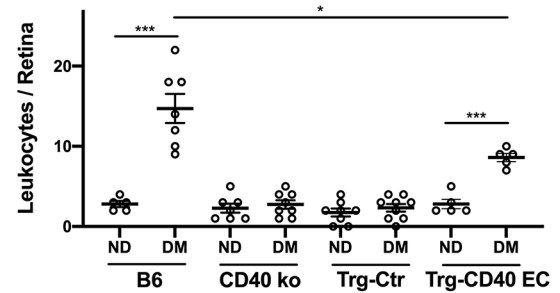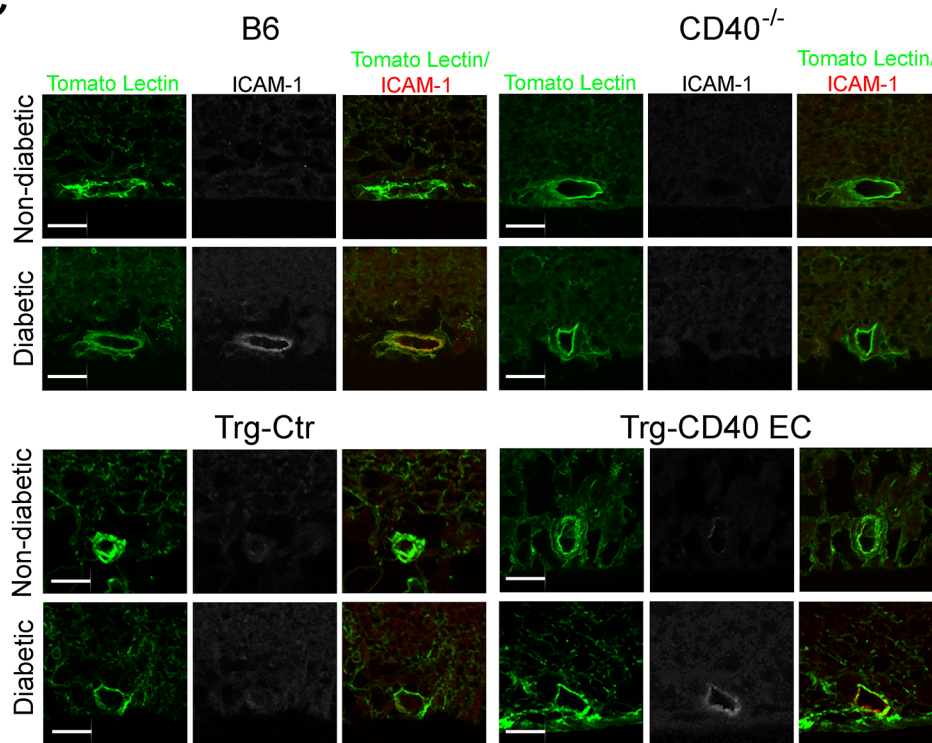
FIGURE 1. **CD40 expression in endothelial cells from diabetic mice promotes upregulation of ICAM-1 and leukostasis in the retina**. (**A**) Double transgenic (Trg-CD40 EC) mice express CD40 in endothelial cells. The cartoon depicts the binary tetracycline (Tet) repressible system. Immunohistochemistry for CD40 in the retinas of B6, CD40$^{-/-}$, single transgenic (Trg-Ctr), or double transgenic (Trg-CD40 EC) mice. Sections were incubated with biotinylated anti-CD40 mAb followed by incubation with streptavidin-Alexa Fluor 647 and co-staining with Tomato lectin-DyLight 488 (X200). Bar, 10 µm. Four animals per group were analyzed. Results are representative of three independent experiments. (**B**) At 2 months of diabetes, retinas from diabetic B6, CD40$^{-/-}$, Trg-Ctr, and Trg-CD40 EC mice as well as from non-diabetic controls were collected and used for mRNA extraction. The mRNA levels of ICAM-1 were assessed by real time quantitative PCR using 18S rRNA as internal control. One non-diabetic B6 mouse was given an arbitrary value of one and data are expressed as fold-increase compared

to this animal. There were seven to nine animals per group. (**C**) At 2 months of diabetes, retinal sections were incubated with anti-ICAM-1 mAb plus either Tomato Lectin. GCL = Ganglion cell layer; IPL = Inner plexiform layer; INL = Inner nuclear layer. Scale bar, 10 μm. 6 mice/group. (**D**) At 2 months of diabetes, adherent leukocytes in the retinal vasculature of diabetic and non-diabetic control mice were quantified by labeling with Concanavalin A. *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$ by ANOVA.

addition, mRNA levels of NOS2 were increased in the retinas of diabetic B6 mice (Fig. 2D). In contrast, rescue of CD40 expression in endothelial cells failed to upregulate NOS2 levels in diabetic animals (see Fig. 2A). Diabetic mice did not exhibit detectable upregulation of IL-6 and CXCL1 mRNA levels (Figs. 2E, 2F). Altogether, CD40 expressed in retinal endothelial cells drives upregulation of ICAM-1 but not TNF-α, IL-1β, and NOS2 in the diabetic retina.

CD40-Activated Retinal Endothelial Cells Do Not Secrete TNF-α and IL-1β or Upregulate NOS2

Retinal endothelial cells do not secrete TNF-α in response to CD40 stimulation.[13] We determined whether these cells release IL-1β after CD40 ligation. Given that primary retinal endothelial cells have low expression of CD40 under basal conditions,[7,11] these cells were transduced with a CD40-encoding retroviral vector, as previously described,[7,15] in order to optimally assess the functional effects of CD40 ligation (Fig. 3A). This approach results in expression of functional CD40 because strong ICAM-1 upregulation was detected in response to CD154 stimulation (Fig. 3B). Incubation of CD40-expressing retinal endothelial cells with CD154 not only failed to stimulate release of TNF-α but also IL-1β (Fig. 3C). In contrast, CD154 caused marked upregulation of CCL2 secretion (Fig. 3C). Thus, retinal endothelial cells failed to secrete not only TNF-α but also IL-1β in response to CD40 stimulation. These results are similar to those reported in retinal Müller cells.[9] Moreover, incubation with high glucose conditions did not enable retinal endothelial cells to secrete TNF-α or IL-1β. Whereas high glucose conditions increased CD40 expression in retinal endothelial cells and potentiated upregulation of ICAM-1 in response to CD154, no TNF-α or IL-1β were detected under these conditions (Fig. 3D). Next, we examined the effects of CD40 ligation on NOS2 upregulation. Stimulation with CD154 did not upregulate NOS2 in retinal endothelial cells, whereas, as previously reported,[8] NOS2 expression was increased in CD40-stimulated Müller cells (Fig. 3E).

CD40-Activated Retinal Endothelial Cells Do Not Secrete Extracellular ATP or Cause TNF-α and IL-1β Production in Bystander Myeloid Cells

Similar to retinal endothelial cells, retinal Müller cells do not secrete detectable amounts of TNF-α and IL-1β in response to CD40 stimulation.[9] However, studies in transgenic mice with CD40 expression restricted to Müller cells revealed that the presence of CD40 in these cells enabled upregulation of retinal TNF-α, IL-1β mRNA levels after induction of diabetes.[9] The explanation for the ability of Müller cell CD40 to upregulate these cytokines is that ligation of CD40 in Müller cells triggers release extracellular ATP that enables bystander myeloid cells to secrete these pro-inflammatory cytokines dependent of the purinergic receptor P2X$_7$.[9] Thus, we examined whether the lack of TNF-α/IL-1β upregulation in diabetic Trg-CD40 EC mice might be related to inability of retinal endothelial cells

to release extracellular ATP in response to CD40 ligation. CD40 expressing retinal endothelial cells failed to release extracellular ATP in response to CD154 (Fig. 4A). Next, we explored whether CD40 ligation in retinal endothelial cells induces TNF-α and IL-1β secretion by bystander myeloid cells. We used the CD40-null monocytic cells (MonoMac6) in order to avoid the effects of direct CD40 ligation on these cells, and because MonoMac6 have been demonstrated to secrete TNF-α/IL-1β in response to ATP secreted by CD40-activated Müller cells.[9] Endothelial cells were incubated with or without MonoMac6 cells in the presence or absence of CD154. Retinal endothelial cells, MonoMac6 cells and the combination of these two cell types failed to secrete TNF-α and IL-1β in response to CD154 (Figs. 4B, 4C). Taken together, CD40 ligation in retinal endothelial cells does not result in secretion of extracellular ATP and does not trigger production of TNF-α and IL-1β by myeloid cells.

CD40 Expressed in Endothelial Cells Does Not Significantly Increase P2X$_7$ mRNA Levels in the Diabetic Retina

Upregulation of the P2X$_7$ receptor accompanies and facilitates in vivo purinergic signaling. Moreover, in the presence of diabetes, B6 mice and transgenic mice that express CD40 restricted to retinal Müller cells upregulate P2X$_7$ mRNA levels, and P2 × 7 is required for upregulation of TNF-α, IL-1β, and NOS2.[9] In contrast to diabetic B6 mice, P2X$_7$ mRNA levels were not increased in the retinas of diabetic Trg-CD40 EC mice (Fig. 5). These findings suggest that expression of CD40 in endothelial cells is unable to modulate P2X$_7$ signaling in vivo in the diabetic retina.

DISCUSSION

CD40 is an upstream inducer of various inflammatory responses in experimental diabetic retinopathy.[8,9] We report that the expression of CD40 restricted to endothelial cells in diabetic mice is sufficient to induce upregulation of ICAM-1 in retinal endothelial cells, an event considered important in the pathogenesis of this disease. This response is likely a direct consequence of CD40 signaling because CD40 stimulation in vitro triggers upregulation of ICAM-1 in retinal endothelial cells. In contrast, expression of CD40 restricted to endothelial cells is unable to upregulate TNF-α, IL-1β, and NOS2 in the diabetic retina. This finding is explained not only by the inability of retinal endothelial cells to directly produce these pro-inflammatory cytokines after CD40 ligation but, importantly, by the inability of these cells to secrete extracellular ATP and induce purinergic-dependent pro-inflammatory cytokine production in by-stander myeloid cells. This work is also important because, to our knowledge, this is the first study that examines the in vivo role in an inflammatory disease of CD40 expressed solely in endothelial cells.

Diabetes increases not only CD40 expression in retinal endothelial cells but also expression of CD154.[8,28−30] In addition to a membrane-bound form, CD154 exists
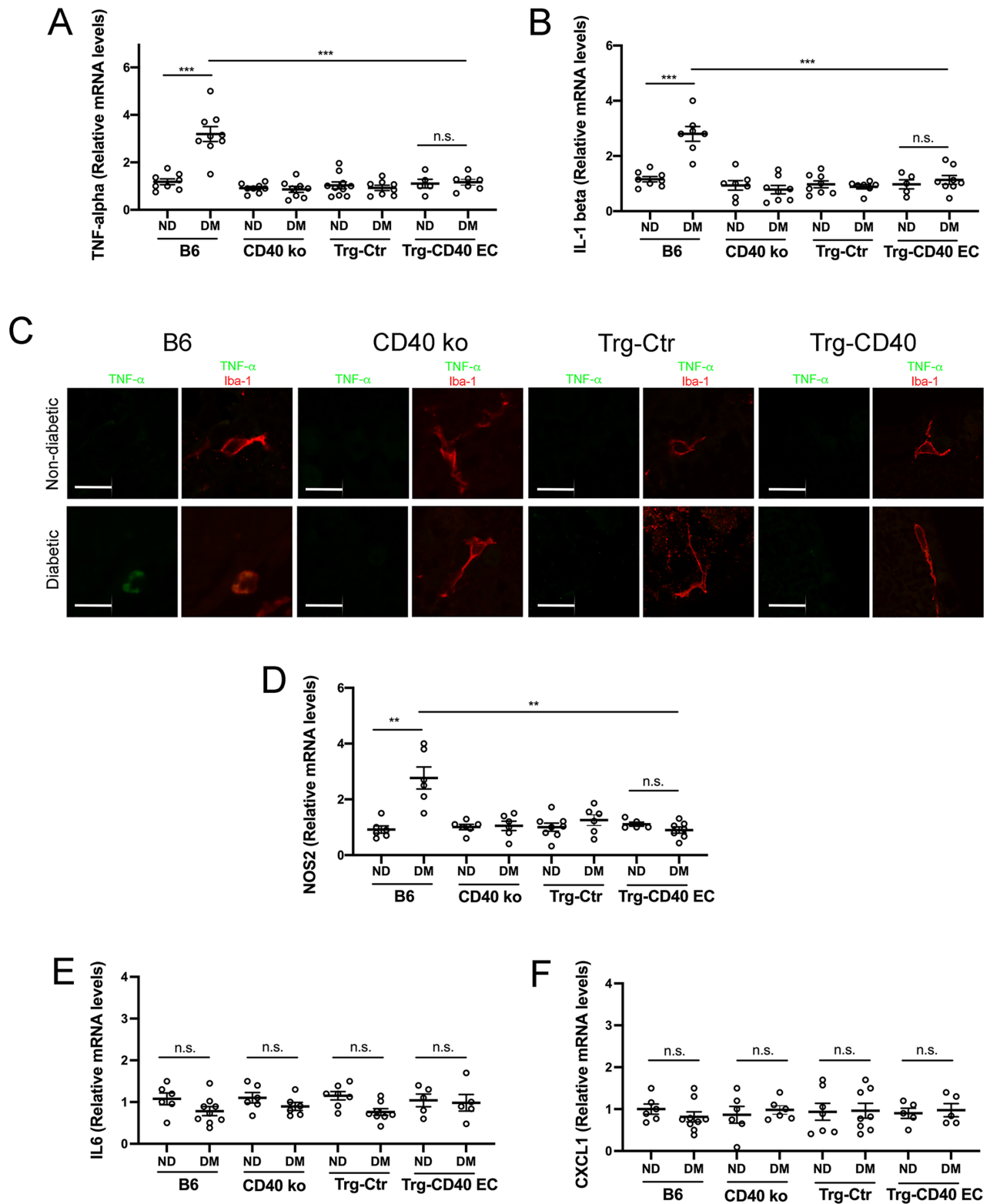
FIGURE 2. **CD40 expression in endothelial cells from diabetic mice does not lead to upregulation of TNF-α, IL-1β, and NOS2 in the retina**. (**A, B**) At 2 months of diabetes, retinas from diabetic B6, CD40$^{-/-}$, Trg-Ctr, and Trg-CD40 EC mice as well as from non-diabetic controls were collected and used for mRNA extraction. The mRNA levels of TNF-α and IL-1β were assessed as above. There were seven to nine animals per group. (**C**) At 2 months of diabetes, retinal sections were incubated with antibodies against TNF-α and anti-Iba-1 (expressed in microglia/macrophages). Scale bar, 10 μm. There were six mice per group. (**D–F**) The mRNA levels of NOS2, IL-6, and CXCL1 at 2 months of diabetes. ******$P < 0.01$; *******$P < 0.001$ by ANOVA. n.s. = not significant.
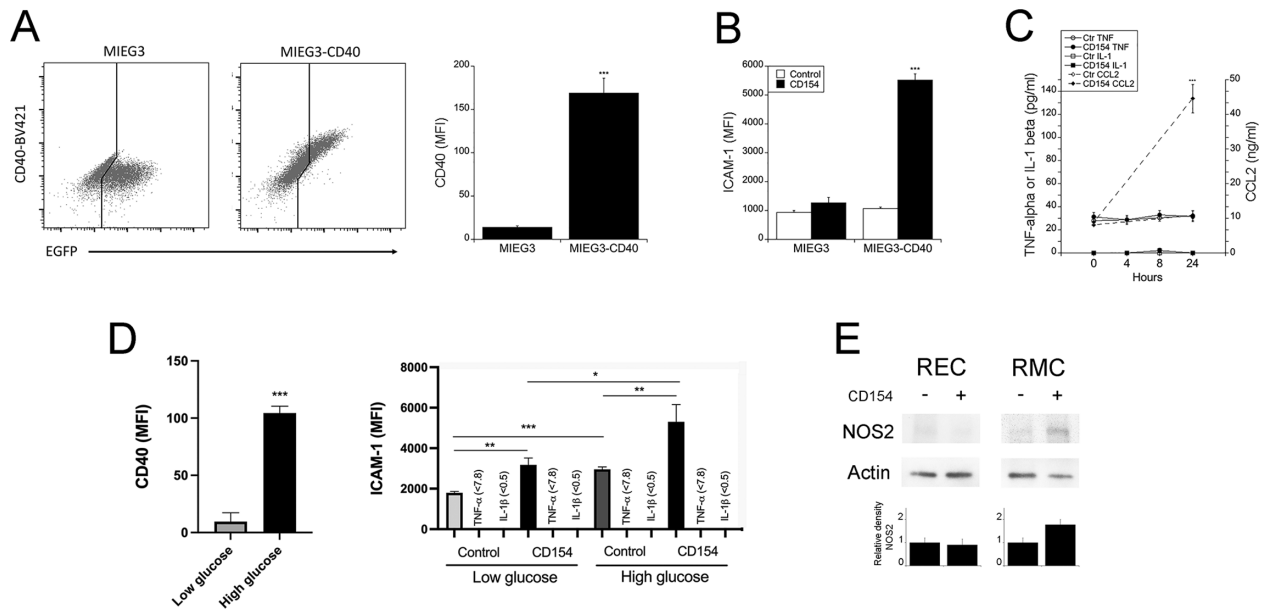
FIGURE 3. **CD40-activated retinal endothelial cells do not secrete TNF-α and IL-1β or upregulate NOS2.** (**A**) Human retinal endothelial cells were transduced with EGFP-encoding empty retroviral vector (MIEG3) or with CD40-encoding retroviral vector (MIEG3-CD40). Dot plot shows expression of EGFP and CD40. (**B**) Endothelial cells were incubated with or without CD154 and ICAM-1 expression was examined by flow cytometry at 24 hours. (**C**) CD40-expressing retinal endothelial cells were incubated with or without CD154 and concentrations of TNF-α and IL-1β were measured at different time points of in vitro culture. In addition, concentration of CCL2 was examined at 0 and 24 hours. Results are presented as mean ± standard deviation (SD) of triplicate wells and are representative of three independent experiments. (**D**) Human retinal endothelial cells were incubated in low glucose or high glucose conditions for 7 days. CD40 expression was examined by flow cytometry. Retinal endothelial cells were incubated with or without CD154. ICAM-1 expression was examined by flow cytometry at 24 hours. Concentrations of TNF-α and IL-1β were measured by ELISA at 4 hours and 24 hours, respectively, and were found to be <7.8 pg/mL and < 0.5 pg/mL, respectively. (**E**) CD40-expressing retinal endothelial cells (RECs) or retinal Müller cells (RMCs) were incubated with or without CD154. Expression of NOS2 and actin were examined at 24 hours by immunoblot. Results are presented as mean ± SD of triplicate samples and are representative of three independent experiments. ***$P < 0.001$ by Student's t-test.
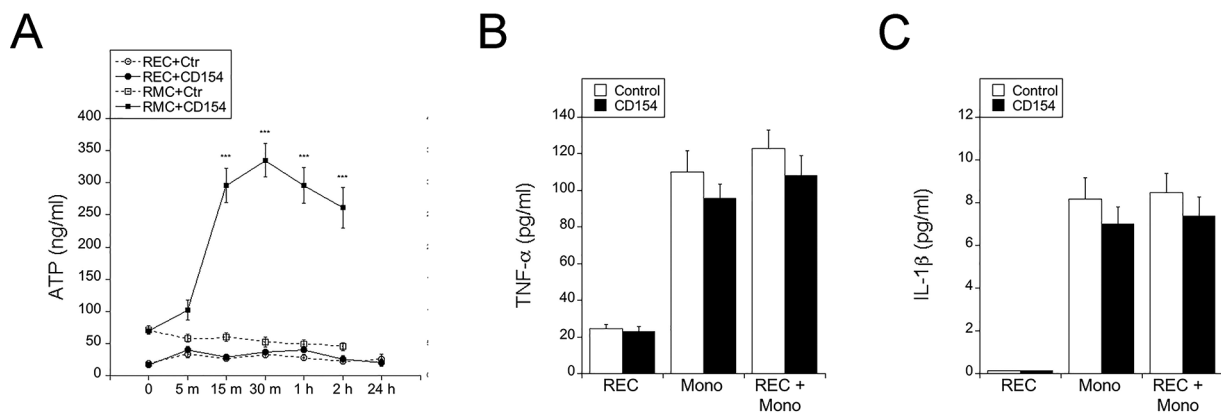

FIGURE 4. **CD40-activated retinal endothelial cells do not secrete ATP or induce production of pro-inflammatory cytokines by monocytic cells.** (**A**) CD40-expressing retinal endothelial cells were incubated with or without CD154 and concentrations of extracellular ATP were measured at different time points. Release of extracellular ATP by CD40⁺ human Muller cells is included for comparison. (**B, C**), CD40-expressing retinal endothelial cells were incubated with CD40⁻ monocytic cells (MonoMac6) with or without CD154. TNF-α **B** and IL-1β **C** were measured in supernatants by ELISA. Results are presented as mean ± SD of triplicate wells and are representative of three independent experiments. ***$P < 0.001$ by Student's t-test.

as a as a biologically active soluble homotrimer present in the circulation.[31] Serum levels of soluble CD154 are increased in diabetic mice and in patients with diabetes, particularly those with microangiopathy.[28–30] Importantly, serum CD154 from patients with diabetes induces pro-

inflammatory responses in endothelial cells.[30] In addition to systemic elevation of CD154, there may be a local elevation of CD154 in the diabetic retina given that microthrombosis in retinal capillaries occur in patients and animals with diabetic retinopathy and activated platelets express CD154.[32]
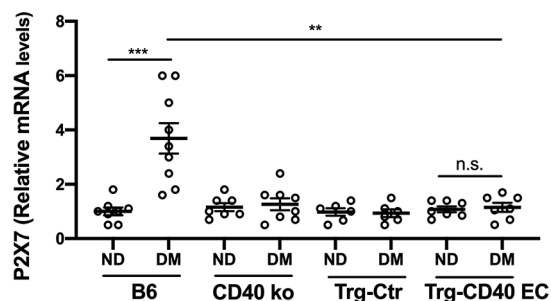
FIGURE 5. CD40 expression in endothelial cells from diabetic mice does not lead to upregulation of $P2X_7$ in the retina. At 2 months of diabetes, retinas from diabetic B6, $CD40^{-/-}$, Trg-Ctr, and Trg-CD40 EC mice as well as from non-diabetic controls were collected and used for mRNA extraction. The mRNA levels were assessed as above. There were seven to nine animals per group. $**P < 0.01$; $***P < 0.001$ by ANOVA.

All these findings would support that the CD40 to CD154 pathway at the level of retinal endothelial cells is activated in diabetes.

The demonstration that CD40 expressed in endothelial cells promotes in vivo upregulation of ICAM-1 in these cells as well as leukostasis is likely relevant to the pathogenesis of vascular pathology. ICAM-1 upregulation has been linked to vascular leakage and the development of capillary degeneration.[22] The latter process consists of the transformation of retinal capillaries into tubes of basement membrane due to the death of vascular cells. This process is relevant because degenerate retinal capillaries lack blood flow, likely contributing to retinal ischemia and subsequent neovascularization. In addition, the increased interaction between leukocytes and endothelial cells may lead to capillary closure, death of endothelial cells, and development of degenerate capillaries.[33] Taken together, despite their relatively restricted nature, inflammatory responses driven by endothelial cell CD40 are likely to contribute to the development of diabetic retinopathy.

Studies in transgenic mice with expression of CD40 restricted to Müller cells uncovered purinergic signaling as a pathway that allows CD40 expressed in non-hematopoietic cells to induce TNF-α and IL-1β upregulation in myeloid cells.[9] Similar to retinal endothelial cells, Müller cells are unable to directly produce significant amounts of TNF-α and IL-1β in response to CD40 stimulation.[9] However, in contrast to retinal endothelial cells, Müller cells activate bystander myeloid cells to produce these pro-inflammatory cytokines via a mechanism dependent on ATP secretion by Müller cells and engagement $P2X_7$ in myeloid cells.[9] These results suggest that the expression of CD40 in retinal endothelial cells would result in a localized pattern of inflammatory responses, whereas CD40 in Müller cells triggers inflammatory responses in Müller cells and myeloid cells. In addition, given that TNF-α and IL-1β stimulate ICAM-1 and NOS2 expression,[34,35] the lack of TNF-α and IL-1β upregulation in diabetic Trg-CD40-EC mice may explain why ICAM-1 upregulation and leukostasis in these mice appeared to be still lower than those observed in diabetic B6 mice, and why diabetic Trg-CD40 EC mice do not upregulate NOS2. These findings support that Müller cells play a more generalized role in driving inflammation in the diabetic retina compared to retinal endothelial cells.

Endothelial cells from different vascular beds are functionally heterogeneous, a characteristic that can affect their responses to inflammatory stimuli.[36,37] For example, human aortic endothelial cells but not human retinal endothelial cells produce TNF-α in response to CD40 stimulation, and upregulate CX3CL1 when incubated with either TNF-α or CD154.[13] Whereas retinal endothelial cells did not secrete extracellular ATP and recruit bystander myeloid cells to secrete pro-inflammatory cytokines, it is possible that endothelial cells in other organs may be able to do so. Such a demonstration may be relevant to the pathogenesis of CD40-driven diseases where purinergic signaling also appears to play a pro-inflammatory role, such as inflammatory bowel disease, atherosclerosis, and lupus nephritis.[38–40]

In summary, this study revealed that expression of CD40 in endothelial cells is sufficient to trigger an important inflammatory response in the retina of diabetic mice. These findings support the central role of CD40 as an upstream regulator of inflammation in the diabetic retina. CD40 is upregulated not only in retinal endothelial and Müller cells but also in microglia/macrophages in diabetic mice.[8] Given that CD40 ligation induces pro-inflammatory responses in microglia/macrophages,[41] it is possible that CD40 signaling at the level of these cells also participates in the development of retinal inflammation in diabetes. Nevertheless, the studies herein and those conducted in mice that express CD40 restricted to Müller cells[9] revealed that expression of CD40 in non-hematopoietic cells is enough to induce inflammation in vivo.

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