Retina

CD40 Upregulation in the Retina of Patients With Diabetic Retinopathy: Association With TRAF2/TRAF6 Upregulation and Inflammatory Molecule Expression

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Purpose. CD40 is upregulated in the retinas of diabetic mice, drives pro-inflammatory molecule expression, and promotes diabetic retinopathy. The role of CD40 in diabetic retinopathy in humans is unknown. Upregulation of CD40 and its downstream signaling molecules TNF receptor associated factors (TRAFs) is a key feature of CD40-driven inflammatory disorders. We examined the expression of CD40, TRAF2, and TRAF6 as well as pro-inflammatory molecules in retinas from patients with diabetic retinopathy.

Methods. Posterior poles from patients with diabetic retinopathy and non-diabetic controls were stained with antibodies against von Willebrand factor (labels endothelial cells), cellular retinaldehyde-binding protein (CRALBP), or vimentin (both label Müller cells) plus antibodies against CD40, TRAF2, TRAF6, ICAM-1, CCL2, TNF-α, and/or phospho-Tyr783 phospholipase Cγ1 (PLCγ1). Sections were analyzed by confocal microscopy.

Results. CD40 expression was increased in endothelial and Müller cells from patients with diabetic retinopathy. CD40 was co-expressed with ICAM-1 in endothelial cells and with CCL2 in Müller cells. TNF-α was detected in retinal cells from these patients, but these cells lacked endothelial/Müller cell markers. CD40 in Müller cells from patients with diabetic retinopathy co-expressed activated phospholipase Cγ1, a molecule that induces TNF-α expression in myeloid cells in mice. CD40 upregulation in endothelial cells and Müller cells from patients with diabetic retinopathy was accompanied by TRAF2 and TRAF6 upregulation.

Conclusions. CD40, TRAF2, and TRAF6 are upregulated in patients with diabetic retinopathy. CD40 associates with expression of pro-inflammatory molecules. These findings suggest that CD40-TRAF signaling may promote pro-inflammatory responses in the retinas of patients with diabetic retinopathy.

Keywords: CD40, TRAF, Mueller cell, endothelial cell, diabetic retinopathy

It is estimated that there were 451 million patients with diabetes worldwide in 2017, and the prevalence of diabetes will continue to increase to 693 million patients by 2040.[1] Approximately 35% of these patients develop diabetic retinopathy,[2] a complication that is the most common cause of blindness in working-age adults in developed countries.[3] In the early nonproliferative stages of diabetic retinopathy, patients exhibit retinal changes that include microaneurysms and capillary degeneration, leading to areas of nonperfusion.[4] In proliferative diabetic retinopathy, the subsequent stage of the disease, the ischemia-driven expression of angiogenic factors, such as vascular endothelial growth factor (VEGF) leads to retinal neovascularization.[5] Furthermore, patients with proliferative diabetic retinopathy can also develop a fibrovascular epiretinal membrane that can result in retinal detachment and visual loss.

Whereas the pathogenesis of diabetic retinopathy is multifactorial, low-grade chronic inflammation plays an important role in the development of this disease.[6–8] Increased retinal expression of inflammatory molecules, including ICAM-1, TNF-α, and CCL2, is associated with diabetic retinopathy in humans.[9–14] Moreover, experimental evidence in animals revealed that low-grade chronic inflammation contributes to the development of diabetic retinopathy.[6–8] Upregulation of ICAM-1 in retinal endothelial cells promotes leukocyte adherence, and the disruption of ICAM-1 – CD18 interaction in diabetic mice reduces the development of degenerate capillaries.[15] Retinal microglia/macrophages in diabetic mice express TNF-α,[16] a cytokine that promotes capillary degeneration.[17] Moreover, administration of an anti-TNF-α agent reduced leukocyte adhesion in the retina and breakdown of the blood-retinal barrier in diabetic rats.[18] CCL2 promotes vascular permeability and monocyte/macrophage recruitment into the retina of diabetic mice.[19] This is further supported by experimental evidence that administration of a dual

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inhibitor of CCR2 (receptor for CCL2) and CCR5 reduced both phenomena in the retina of diabetic mice.[20]

Therapeutic approaches for diabetic retinopathy include intravitreal administration of anti-VEGF agents, laser photocoagulation, and steroids.[21,22] However, a large proportion of patients have an inadequate response to anti-VEGF agents, and the current treatment options have significant side effects.[23–25] As such, the development of novel approaches to the treatment of diabetic retinopathy is a critical area of research. Identification of the upstream events that trigger inflammatory responses in diabetic retinopathy may lead to development of such novel approaches.

CD40 is a central driver of retinal inflammation and the development of retinopathy in diabetic mice.[26] CD40 is a member of the TNF receptor superfamily that is upregulated in retinal endothelial cells, Müller cells, and microglia/macrophages in mice with experimental diabetic retinopathy.[26] Diabetic $CD40^{-/-}$ mice do not upregulate ICAM-1, TNF-α, IL-1β, and CCL2 in the retina and do not develop diabetic retinopathy.[26–28] Studies in transgenic $CD40^{-/-}$ mice with rescue of CD40 restricted to Müller cells or endothelial cells revealed that expression of CD40 isolated to these cells is sufficient to drive inflammatory responses in diabetic mice.[28–30] Diabetic mice that express CD40 restricted to endothelial cells exhibit ICAM-1 upregulation in these cells and develop leukostasis.[29] This finding is consistent with the demonstration that ligation of CD40 triggers upregulation of ICAM-1 in retinal endothelial cells.[27] Diabetic mice with expression of CD40 restricted to Müller cells upregulate CCL2 in these cells[28,30] in agreement with the ability of CD40 stimulation to induce CCL2 production in retinal Müller cells.[27] Importantly, the presence of CD40 in Müller cells also triggers pro-inflammatory molecule expression in bystander microglia/macrophages.[28,30] CD40 ligation in Müller cells induces phospholipase C γ1 (PLCγ1)-dependent release of extracellular ATP that engages the purinergic receptor $P2X_7$ expressed in bystander microglia/macrophages, enabling these cells to secrete TNF-α and IL-1β.[28] Moreover, expression of CD40 restricted to Müller cells in diabetic mice is sufficient for development of early diabetic retinopathy in mice.[28,30]

CD40 functions by recruiting TNF receptor associated factors (TRAFs), of which TRAF2 and TRAF6 are major mediators of the effects of CD40.[31] Indeed, disruption of CD40-TRAF2 or CD40-TRAF6 signaling is sufficient to markedly inhibit in vitro pro-inflammatory responses in retinal cells.[27] Moreover, in contrast to diabetic $CD40^{-/-}$ mice rescued to express wild type (WT) CD40 in Müller cells, diabetic mice rescued with CD40 that cannot recruit TRAF2 or TRAF6 do not upregulate ICAM-1, TNF-α, IL-1β, CCL2, or $P2X_7$ in the retina.[30] Disruption of CD40-TRAF2 signaling in Müller cells prevents the development of diabetic retinopathy.[30] Importantly, intravitreal administration of a cell-permeable peptide that blocks CD40-TRAF2 signaling markedly impaired upregulation of ICAM-1, TNF-α, IL-1β, CCL2, and $P2X_7$ in the retina as well as reduced retinal leukostasis in diabetic B6 mice.[30] These findings support that CD40 is a therapeutic target against diabetic retinopathy.

The levels of CD40 expression are low under basal conditions.[32,33] However, induction or upregulation of CD40 expression is a key feature of inflammatory disorders driven by CD40.[33,34] Furthermore, increased expression of TRAF appears to correlate with the TRAF pathways that mediate pro-inflammatory responses induced by CD40.[35] Thus, identification of the cell types that upregulate CD40 and TRAFs in diabetic retinopathy can provide an indication of the cells in which CD40-TRAF signaling is likely activated.

Whereas studies in mice support the central role of CD40 in the development of diabetic retinopathy, little is known about the relevance of retinal CD40 in patients with this disease. The studies herein were conducted to determine whether the expression of CD40, TRAF2, and/or TRAF6 are increased in the retinas of patients with diabetic retinopathy and whether CD40 co-localizes with pro-inflammatory molecules in the retinas of these patients.

Table 1. Antibodies Used for Immunofluorescence Microscopy

Marker	Antibody	Source	Dilution
CRALBP	15356-1-AP	Proteintech Group, Rosemont, IL, USA	1:200 (in vivo) 1:2000 (in vitro)
Vimentin	NB300-223	Novus Biologicals, Littleton, CO, USA	1:300 (in vivo) 1:3000 (in vitro)
von Willebrand Factor	GTX74137	GeneTex, Irvine, CA, USA	1:30 (in vivo) 1:1000 (in vitro)
CD40	334302 (5C3)	BioLegend, San Diego, CA, USA	1:50 (in vivo) 1:1000 (in vitro)
CD40 (biotinylated)	334343 (5C3)	BioLegend, San Diego, CA, USA	1:50 (in vivo) 1:1000 (in vitro)
TRAF2	GTX89120	GeneTex, Irvine, CA, USA	1:100 (in vivo) 1:3000 (in vitro)
TRAF6	GTX113029	GeneTex, Irvine, CA, USA	1:100 (in vivo) 1:2000 (in vitro)
ICAM-1	GTX100450	GeneTex, Irvine, CA, USA	1:100 (in vivo) 1:100 (in vitro)
CCL2	14-7096-81 (2H5)	Invitrogen, Waltham, MA, USA	1:25 (in vivo) 1:100 (in vitro)
TNF-α	NBP1-19532	Novus Biologicals, Littleton, CO, USA	1:100 (in vivo) 1:200 (in vitro)
Phospho-Tyr 783 PLCγ1 (biotinylated)	BS-3343-R	Bioss, Woburn, MA, USA	1:200 (in vivo) 1:1000 (in vitro)

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Materials and Methods

Human Subjects

Eyes from eight subjects with documented diabetic retinopathy and three non-diabetic control individuals (1 eye per donor) were obtained postmortem through Eversight (Cleveland, OH, USA). Subjects did not have any other known retinal disease. The stage of diabetic retinopathy was determined by reviewing available clinical information. Four diabetic subjects carried a diagnosis of proliferative diabetic retinopathy. No information about the stage of the disease was available in the remaining four subjects. Eyes were fixed in 4% paraformaldehyde within 16 hours after death. Anterior segments and the vitreous were removed from the eye cups. Posterior poles were maintained in paraformaldehyde for more than 24 hours. The use of human material was in accordance with the Declaration of Helsinki on the use of human material for research.

Immunohistochemistry

Tissues were placed in 30% sucrose followed by embedding in OCT in a mold. Tissues were flash frozen with liquid nitrogen and stored at −80°C. Frozen tissues were sectioned (10 µm) on a Leica cryostat, and the sections were mounted on SuperPlus Slides. Sections of peripheral retinas were incubated with the antibodies listed in Table 1. Fluorescent secondary antibodies were from Jackson ImmunoResearch Laboratories (West Grove, PA, USA). Retinas were analyzed blindly using Olympus FV1200 IX-83 confocal microscope (Oberkochen, Germany). Images were obtained at a total magnification of 400 times. Microscope settings were kept constant for all samples. Images were processed in Photoshop CC 19.1.1. using similar linear adjustments for all samples. Semiquantitative assessment of CD40, TRAF2, and TRAF6 expression in retinal endothelial cells and Müller cells was performed using MetaMorph (Nashville, TN, USA). Briefly, images were thresholded to identify von Willebrand factor^{+} (endothelial cells), CRALBP^{+}, and vimentin^{+} (Müller cells) areas. Pixel intensity for CD40, TRAF2, and TRAF6 in the selected areas were measured.

Cells

The human Müller cell line MIO M1 (gift from Dr. Gloria Limb; University College London, London, England, UK) transduced with a retroviral vector that encodes human CD40 or an empty vector were described previously.[26,28] Primary human retinal endothelial cells were obtained from Cell Systems (Kirkland, WA, USA). In certain experiments, cells were treated with multimeric human CD154 (CD40 ligand; gift from Dr. Richard Kornbluth, Multimeric Biotherapeutics Inc., La Jolla, CA, USA) or a nonfunctional CD154 mutant (T147N) as control.[12] The human monocytic cell line Monomac6 cells (gift from Rene de Waal Malefyt, DNAX Research Institute, Palo Alto, CA, USA) were incubated with or without IFN- γ (100 IU/mL; PeproTech, Rocky Hill, NJ, USA) plus LPS (100 ng/mL; Sigma Aldrich, St. Louis, MO, USA). MonoMac6 cells were stained with anti-TNF-α antibody in the presence or absence of a specific blocking peptide (NBP1-19532PEP; Novus, Littleton, CO, USA). Cells were transfected with non-targeting siRNA or siRNA against human TRAF2 or TRAF6 obtained from Horizon Discovery (Cambridge, UK). Cells were also transfected with siRNA against ICAM-1 (AGCGGAAGAUCAAGAAAUA) or CCL2 (CCAUGGACCACCUGGACAA). Transfections were performed using 50 nM of siRNA and *Trans*IT-X2 (Mirus, Madison, WI, USA).

Immunoblot

Membranes were probed with antibodies to TRAF2, TRAF6, ICAM-1, CCL2 (see Table 1), or actin (sc47778; Santa Cruz Biotechnologies, Santa Cruz, CA, USA), followed by incubation with secondary antibody conjugated to horseradish peroxidase (Santa Cruz Biotechnologies).

Statistics

Results were expressed as the mean ± SEM. Statistical significance was analyzed by ANOVA. Although multiple measurements were obtained in each sample, statistical comparison among patient samples was done using the mean values for these measurements for each patient. Differences were considered statistically significant at $P < 0.05$.

Table 2. Summary of Clinical Data

Subject	Diagnosis	Age/Sex	Medical History
1	Diabetic retinopathy	58/M	Gangrene and stroke
2	Diabetic retinopathy	71/M	Myocardial infarction, end-stage kidney disease, gastro-intestinal bleed, hyperlipidemia, and diabetic neuropathy
3	Diabetic retinopathy	73/F	Myocardial infarction, heart failure, hypertension, and hyperlipidemia
4	Diabetic retinopathy	69/M	Myocardial infarction, ischemic cardiomyopathy, hypertension, stroke, and hyperlipidemia
5	Diabetic retinopathy (Proliferative)	73/M	Gastrointestinal bleed, myocardial infarction, and hyperlipidemia
6	Diabetic retinopathy (Proliferative)	73/M	Myocardial infarction, hypertension, chronic kidney disease, and hyperlipidemia
7	Diabetic retinopathy (Proliferative)	63/M	Coronary artery disease, heart failure, hypertension, and pneumonia
8	Diabetic retinopathy (Proliferative)	58/F	Myocardial infarction, heart failure, chronic kidney disease, and hyperlipidemia
9	Non-diabetic	78/M	Myocardial infarction, hypertension, hyperlipidemia, and alcoholism
10	Non-diabetic	61/M	Myocardial infarction, hyperlipidemia, and hypertension
11	Non-diabetic	53/M	Lung cancer and hypertension

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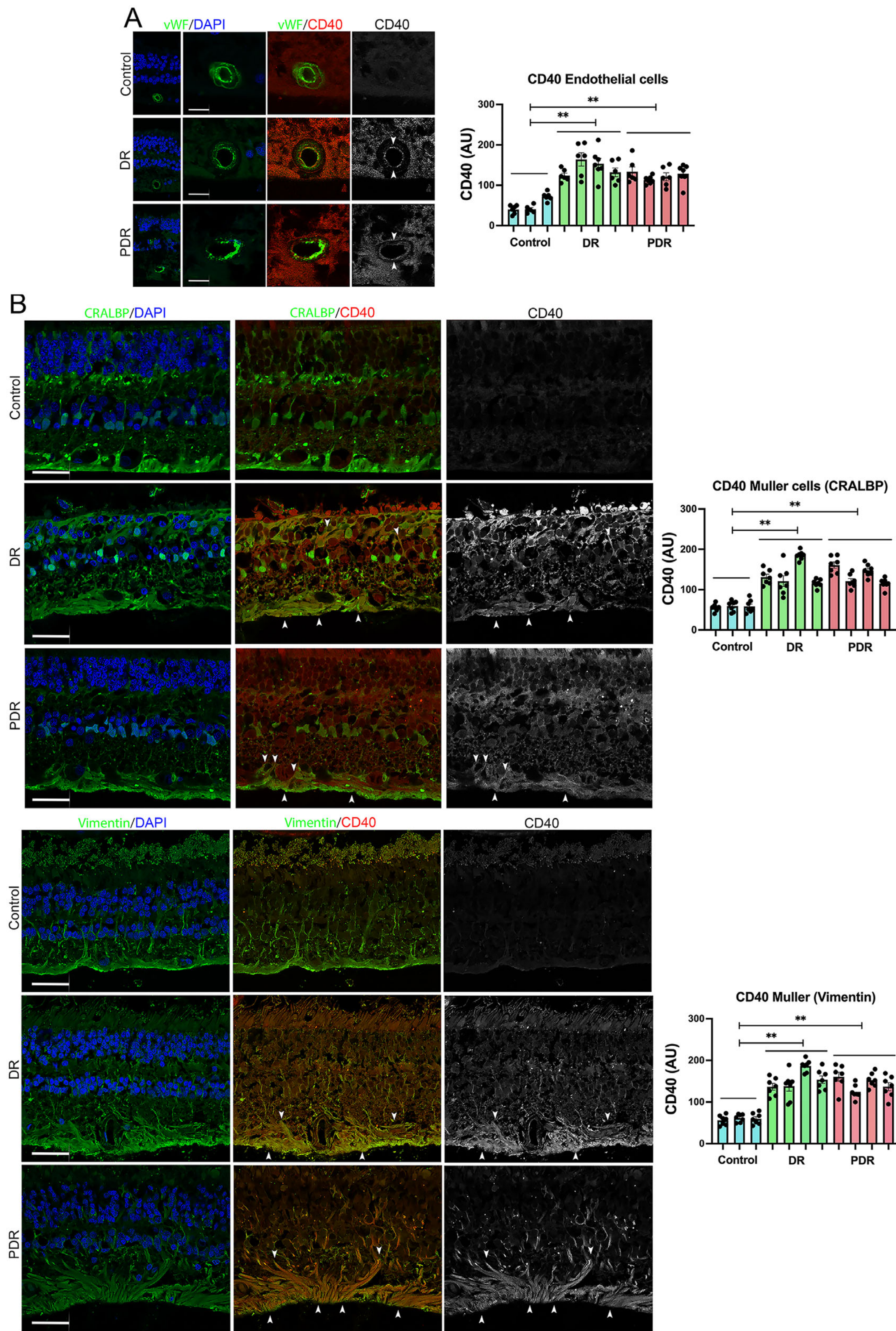

FIGURE 1. **CD40 is upregulated in retinal endothelial cells and Müller cells in patients with diabetic retinopathy.** Posterior poles from patients with diabetic retinopathy (DR, no available information on disease stage), proliferative diabetic retinopathy (PDR), and non-diabetic controls were incubated with anti-CD40 mAb plus antibodies against either: (**A**) von Willebrand factor (vWF, marker of endothelial cells); (**B**) CRALBP; (**C**) Vimentin (both expressed in Müller cells). *Arrowheads* show some of the areas where von Willebrand factor, CRALBP, or Vimentin co-express with CD40. Original magnification times 400. Scale bar, 20 µm (**A**) or 50 µm (**B, C**). Graphs show pixel intensity for CD40 (arbitrary units [AUs]) in von Willebrand factor$^+$, CRALBP$^+$, and Vimentin$^+$ cells. Six to eight fields per subject were analyzed. Statistical comparison among patient samples was done using the mean values for these measurements. $^{**}P < 0.01$ by ANOVA.

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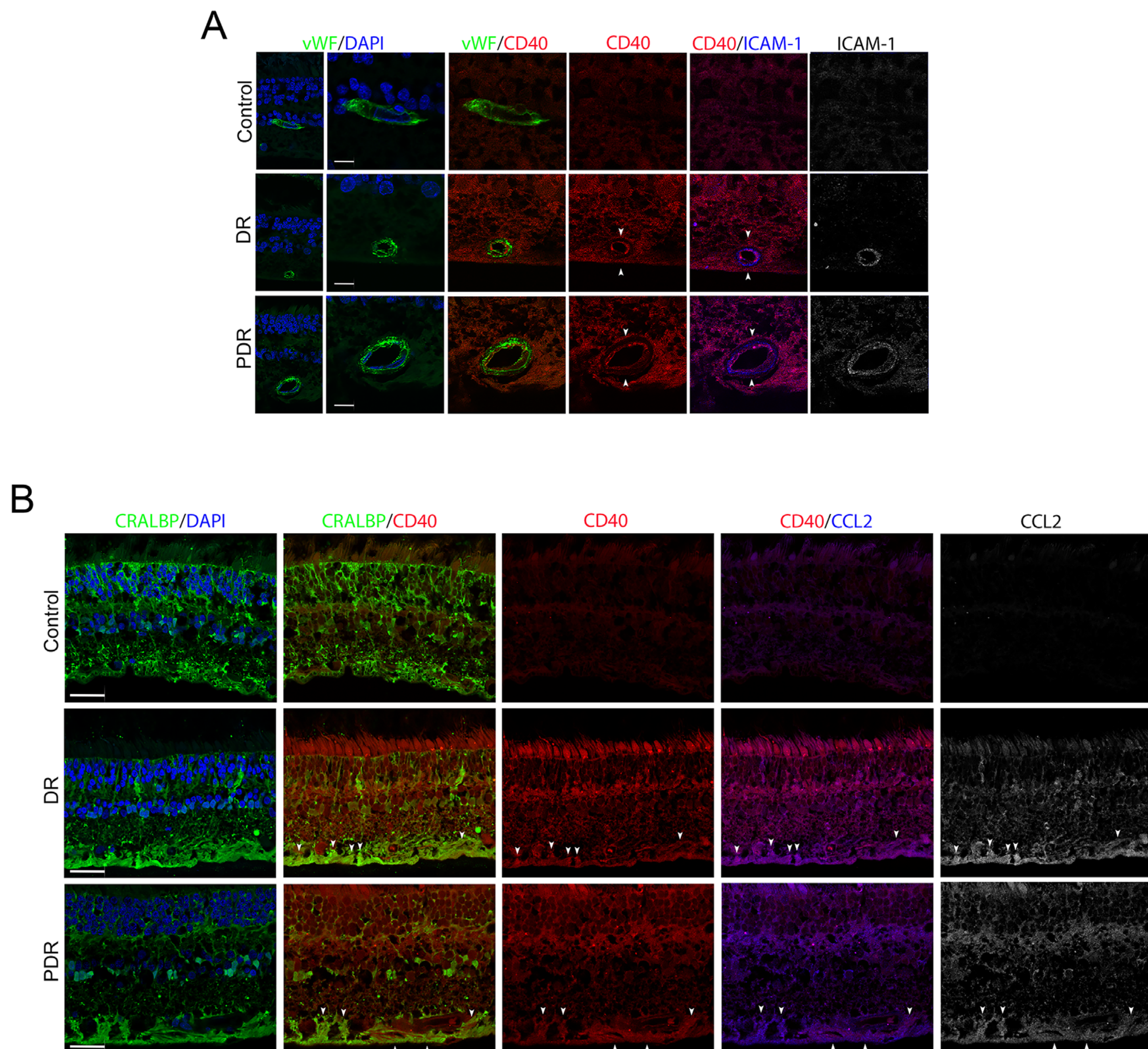

Figure 2. Co-expression of CD40 and pro-inflammatory molecules in the retinas of patients with diabetic retinopathy. Posterior poles from patients with DR, PDR, and non-diabetic controls were incubated with anti-CD40 mAb with either anti-von Willebrand factor plus anti-ICAM-1 antibodies (**A**) or anti-CRALBP plus anti-CCL2 antibodies (**B**). *Arrowheads* show some of the areas where von Willebrand factor or CRALBP co-express with CD40 and ICAM-1 or CCL2. Original magnification times 400. Scale bar, 20 µm (**A**) or 50 µm (**B**).

Results

Expression of CD40 is Increased in Retinal Endothelial Cells and Müller Cells From Subjects With Diabetic Retinopathy

CD40 can be expressed in a broad range of cells that include retinal endothelial cells, Müller cells, and microglia/macrophages.[26,36] CD40 is also detected in various neurons,[37] including ganglion cells in the retina.[36] Whereas CD40 is expressed at low levels in the retina of normal, non-diabetic mice,[26,36] diabetes causes upregulation of CD40 in retinal Müller cells and endothelial cells in mice.[26] Expression of CD40 restricted to either Müller cells or endothelial cells is sufficient to induce inflammatory responses in the retina of diabetic mice.[28–30] Thus, we centered on those cells for our studies in human retinas. We evaluated posterior poles from eight subjects with diabetic retinopathy, four of whom had a history of proliferative diabetic retinopathy. There was no information about the stage of disease in the remaining four subjects and therefore are labelled as simply having diabetic retinopathy. We examined three non-diabetic subjects without a history of retinal disease as controls. Table 2 summarizes the characteristics of the individuals examined. Retinas from these individuals were examined by immunohistochemistry using antibodies that were validated as shown in Supplementary Figure S1. In this regard, anti-CD40 antibodies only stained Müller cells that expressed CD40 but not CD40$^-$ Müller cells; staining with antibodies against TRAF2, TRAF6, ICAM-1, or CCL2 was ablated in cells made deficient in the molecules by transfection with siRNA; and staining with anti-TNF-α antibody was markedly inhibited by incubation with specific blocking peptide (see Supplementary Fig. S1). Control subjects exhibited little immunoreactivity for CD40 in the retina consistent with the low expression

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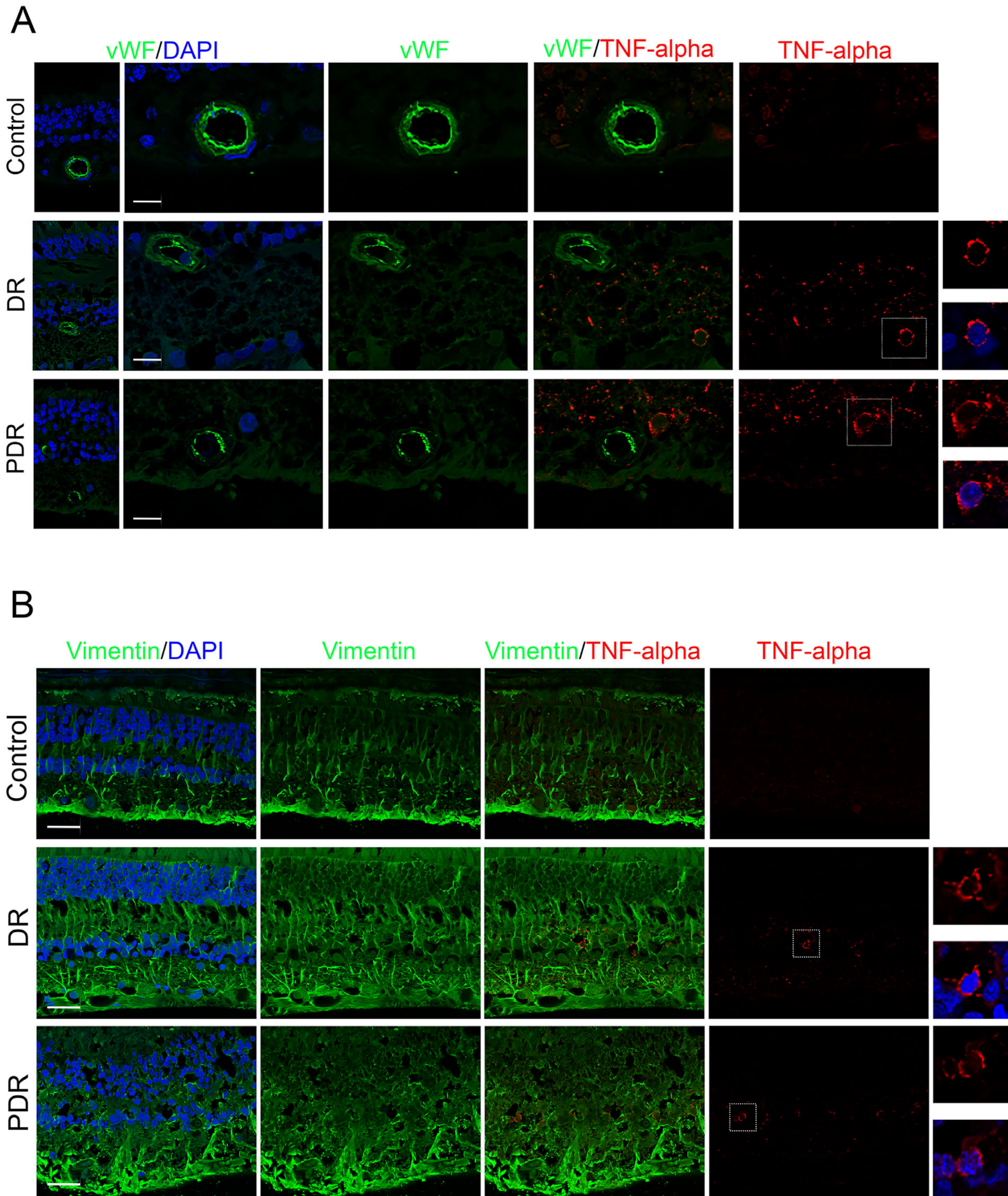

FIGURE 3. **TNF-α does not appear to be expressed in endothelial cells and Müller cells from patients with diabetic retinopathy.** Posterior poles from patients with DR, PDR, and non-diabetic controls were incubated with anti-TNF-α antibody plus either anti-von Willebrand factor (**A**) or anti-vimentin antibody (**B**). TNF-α-expressing cells are shown within boxes. Set of images including von Willebrand factor alone or vimentin alone indicate that TNF-α did not associate with von Willebrand factor or vimentin expression. Original magnification times 400. Scale bar, 20 µm (**A**) or 50 µm (**B**).

of this molecule under basal conditions (Fig. 1). In contrast, CD40 staining was more intense in the retinas from patients with diabetic retinopathy (see Fig. 1). No staining was observed in retinal sections where primary antibodies were omitted (secondary antibodies alone; Supplementary Fig. S2). CD40 expression in patients with diabetic retinopathy was somewhat widespread suggesting expression of CD40 in various retinal cells. Retinal sections were incubated with antibodies against von Willebrand factor to identify retinal endothelial cells and antibodies against CRALBP

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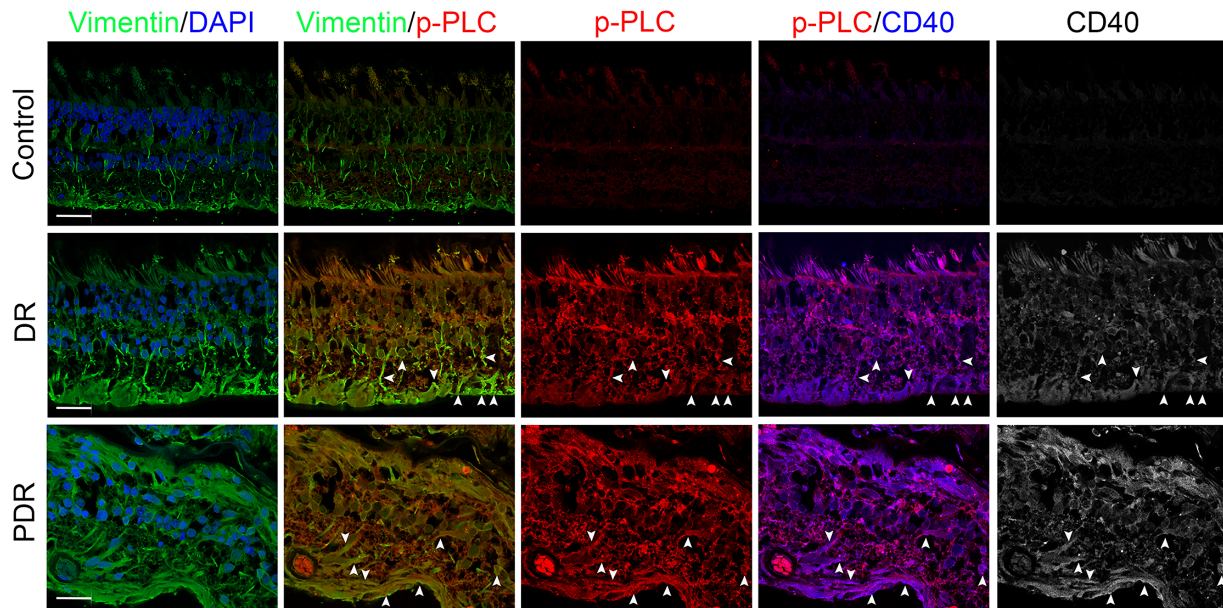

Figure. 4. Patients with diabetic retinopathy expressed activated PLCγ1 in Müller cells. Posterior poles from patients with DR, PDR, and non-diabetic controls were incubated with anti-phospho-Tyr783 PLCγ1 (p-PLC) plus anti-vimentin and anti-CD40 antibodies. *Arrowheads* show some of the areas where phospho-Tyr783 PLCγ1 is co-expressed with vimentin and CD40. Original magnification times 400. Scale bar, 50 μm.

or Vimentin to identify Müller cells. Retinas from control subjects exhibited little immunoreactivity for CD40 in areas that co-expressed von Willebrand factor (retinal endothelial cells; see Fig. 1A). In contrast, CD40 staining was more intense in areas that co-expressed this endothelial cell marker in retinas from patients with diabetic retinopathy (see Fig. 1A). Similarly, compared to retinas from control subjects, there were areas of more intense CD40 staining that co-expressed the Müller cell markers CRALBP or Vimentin in retinas from patients with diabetic retinopathy (see Fig. 1B). These results indicate that CD40 is upregulated in endothelial cells and Müller cells from patients with diabetic retinopathy.

CD40 was Co-Expressed With ICAM-1 and CCL2 in Retinas of Subjects With Diabetic Retinopathy

We examined whether CD40 in retinal cells from subjects with diabetic retinopathy was co-expressed with proinflammatory molecules key to the development of diabetic retinopathy: ICAM-1, CCL2, and TNF-α. As reported,[10] retinal endothelial cells from patients with diabetic retinopathy exhibited increased expression of ICAM-1 (Fig. 2A). Co-staining with anti-CD40 mAb revealed that ICAM-1 was co-expressed with CD40 in retinal endothelial cells from patients with diabetic retinopathy (see Fig. 2A). Compared to Müller cells from control subjects, Müller cells from patients with diabetic retinopathy expressed CCL2 (Fig. 2B). Retinas from these patients revealed areas where this chemokine was co-expressed with CD40 (see Fig. 2B). In addition, we examined expression of TNF-α in retinal endothelial cells and Müller cells. TNF-α was not detected in retinal endothelial cells or Müller cells from control subjects or patients with diabetic retinopathy (Figs. 3A, 3B). Rather, TNF-α was detected in cells that lacked expression of von Willebrand factor and vimentin (see Fig. 3). Commercial antibodies against markers of microglia/macrophages that could be used in combination with the anti-TNF-α antibody were not adequate for identification of microglia/macrophages in the human retinas. Altogether, CD40 was co-expressed with ICAM-1 in retinal endothelial cells and with CCL2 in Müller cells in patients with diabetic retinopathy, whereas these cells did not appear to express TNF-α.

Müller Cells From Patients With Diabetic Retinopathy Expressed Activated PLCγ1

Relevant to the current studies, we previously reported that Müller cells from diabetic mice do not express TNF-α by immunohistochemistry, although this cytokine was detected in retinal microglia/macrophages.[28] Importantly, CD40 present in Müller cells induced protein expression of TNF-α in microglia/macrophages through CD40-driven secretion of ATP by Müller cells and purinergic-dependent production of TNF-α by microglia/macrophages.[28] CD40-dependent expression of activated PLCγ1 in Müller cells is the upstream event that triggers this purinergic pathway in diabetic mice.[28] Thus, we examined whether Müller cells from patients with diabetic retinopathy express activated PLCγ1 that is associated with CD40 expression. Indeed, patients with diabetic retinopathy exhibited detectable immunostaining for phosphor-Tyr783 PLCγ1 (marker of PLCγ1 activation) in Müller cells that co-expressed CD40, whereas those from control subjects did not (Fig. 4).

Retinal Endothelial Cells and Müller Cells From Patients With Diabetic Retinopathy Exhibit Increased Expression of TRAF2 and TRAF6

Increased in vivo expression of not only CD40 but also TRAFs is associated with CD40-driven inflammation.[35]

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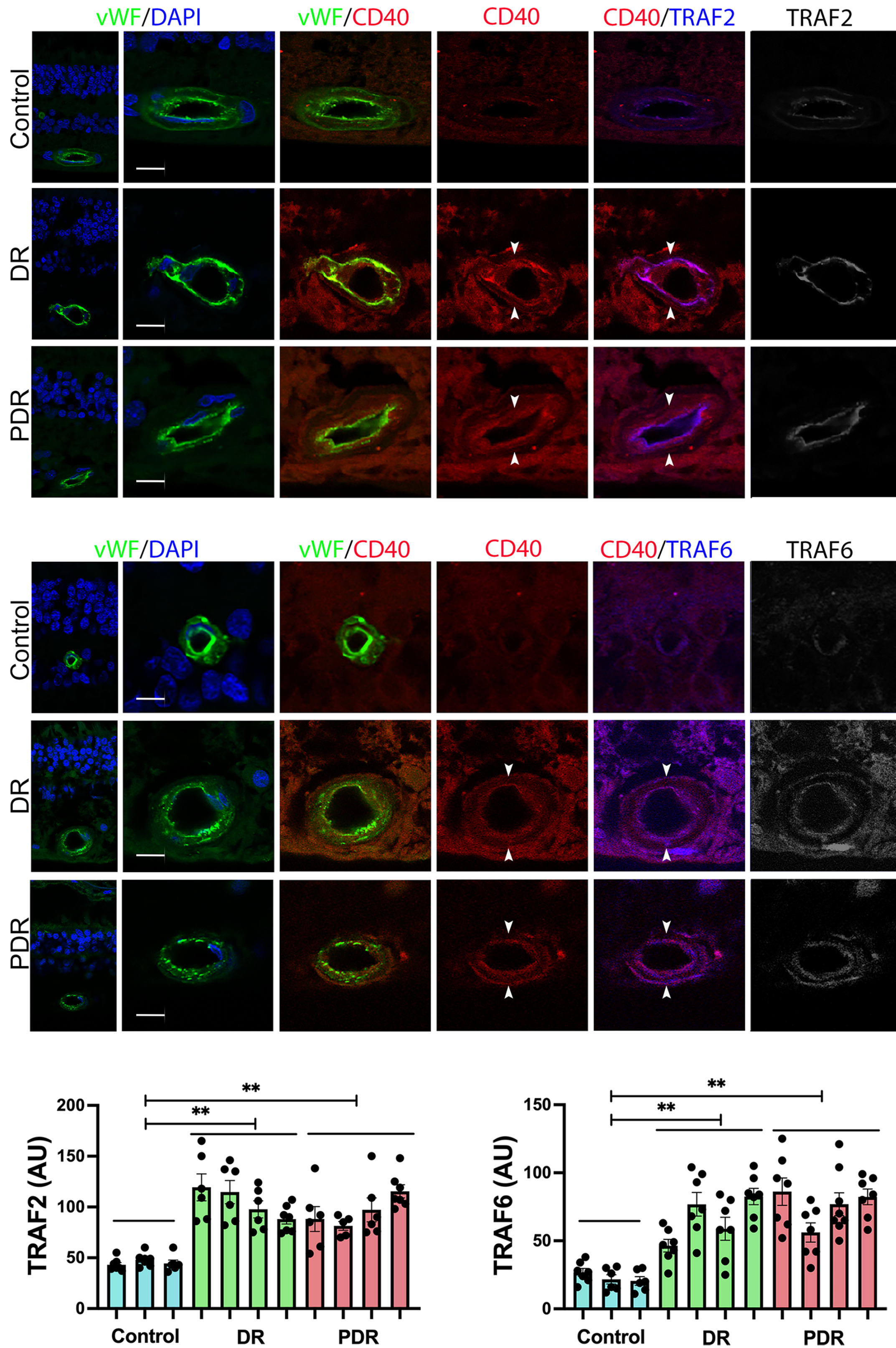

Figure. 5. Patients with diabetic retinopathy exhibit increased expression of TRAF2 and TRAF6 in retinal endothelial cells. Posterior poles from patients with DR, PDR, and non-diabetic controls were incubated with anti-TRAF2 or anti-TRAF6 Abs plus anti-von Willebrand factor antibody. *Arrowheads* show areas where von Willebrand factor is co-expressed with CD40 and TRAF2 or TRAF6. Original magnification times 400. Scale bar, 20 µm. Graphs show pixel intensity (AU) for TRAF 2 or TRAF6 in von Willebrand factor^{+} cells. Six to eight fields per subject were analyzed. Statistical comparison among patient samples was done using the mean values for these measurements. $^{**}P < 0.01$ by ANOVA.

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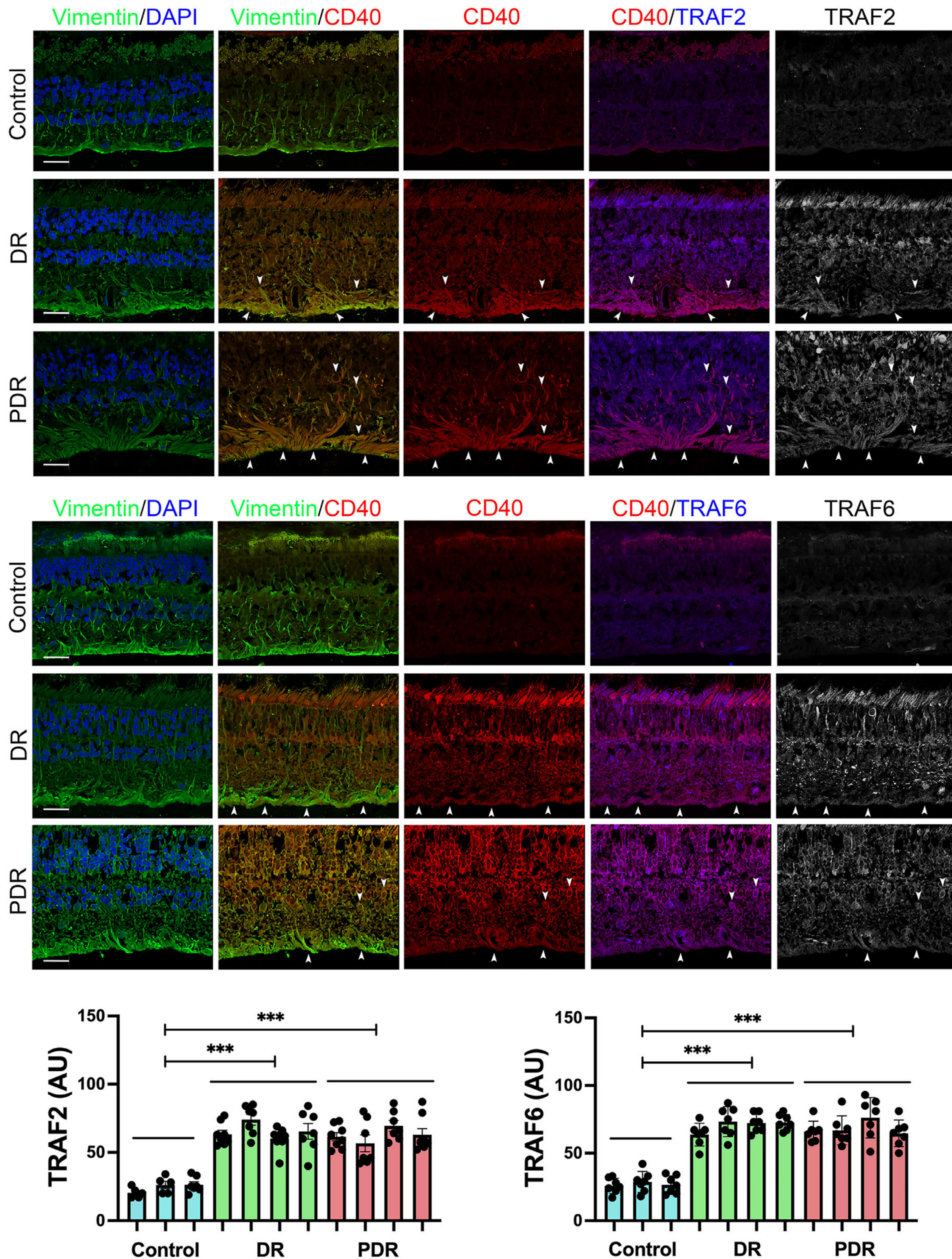

Figure. 6. Patients with diabetic retinopathy exhibit increased expression of TRAF2 and TRAF6 in Müller cells. Posterior poles from patients with DR, PDR, and non-diabetic controls were incubated with anti-TRAF2 or anti-TRAF6 Abs plus anti-vimentin antibody. *Arrowheads* show some of the areas where vimentin is co-expressed with CD40 and TRAF2 or TRAF6. Graphs show signal intensity for TRAF 2 or TRAF6 in vimentin$^+$ cells. Original magnification times 400. Scale bar, 50 µm. Graphs show pixel intensity (AU) for TRAF 2 or TRAF6 in vimentin$^+$ cells. Six to eight fields per subject were analyzed. Statistical comparison among patient samples was done using the mean values for these measurements. ***$P < 0.001$ by ANOVA.

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Control subjects exhibited low immunoreactivity for TRAF2 and TRAF6 in retinal endothelial cells (Fig. 5) and Müller cells (Fig. 6). In contrast, retinal endothelial cells and Müller cells from patients with diabetic retinopathy displayed more intense staining for TRAF2 and TRAF6, and these proteins were co-expressed with CD40 (see Figs. 5, 6).

Discussion

We report that the expression of CD40 in retinal endothelial cells and Müller cells was increased in patients with diabetic retinopathy (see Fig. 7 for graphic summary). These findings are significant because modest upregulation of CD40 in these cells is known to enhance CD40-driven pro-inflammatory molecule expression.[26] Indeed, CD40 was co-expressed with ICAM-1 in endothelial cells and CCL2 in Müller cells from patients with diabetic retinopathy. In addition, these patients expressed activated PLCγ1 in Müller cells. This molecule is a key component of the purinergic pathway that drives expression of pro-inflammatory molecules in the retina of diabetic mice.[28] Finally, retinal endothelial cells and Müller cells from patients with diabetic retinopathy exhibited increased expression of TRAF2 and TRAF6, major mediators of CD40 signaling. These findings suggest that the CD40-TRAF pathway plays an important role in the development of diabetic retinopathy in humans because: (i) upregulation of CD40 is a key feature of CD40-driven inflammatory disorders.[33,34] (ii) CD40 co-localized with ICAM-1 and CCL2, pro-inflammatory molecules that promote diabetic retinopathy and are known to be induced in vitro by CD40 ligation in human cells.[27] (iii) activation of PLCγ1 is the upstream event that drives purinergic-dependent production of pro-inflammatory cytokines, a pathway that CD40 activates in human cells and that promotes diabetic retinopathy in vivo in mice.[28] (iv) TRAF upregulation is associated with CD40-TRAF signaling in vivo,[35] and CD40-TRAF2 as well as CD40-TRAF6 signaling are required for pro-inflammatory molecule upregulation in the retina of diabetic mice.[30]

Relevant to our studies, CD40 expression is increased in renal tubules and infiltrating cells in the kidneys of patients with diabetic nephropathy.[38] In addition, peripheral blood mononuclear cells from patients with poorly controlled type I diabetes exhibit increased mRNA levels of the functional type I isoform of CD40.[39] Outside of diabetes mellitus, CD40 is upregulated in various disorders, including inflammatory bowel disease, systemic lupus erythematosus, rheumatoid arthritis, multiple sclerosis, graft rejection, and atherosclerosis.[33,34,40–45] CD40 promotes inflammation in these diseases, a phenomenon that is likely potentiated by CD40 upregulation because increased levels of CD40 markedly enhances CD154-driven inflammatory responses in vitro.[26] The co-expression of CD40 with pro-inflammatory molecules known to be directly upregulated by CD40 suggests that CD40 promotes inflammatory responses in the retina of patients with diabetic retinopathy.

The expression of CD154 (CD40 ligand) is also altered in diabetes. In addition to membrane CD154 present on activated $CD4^+$ T cells and platelets, there is also a biologically active soluble form of CD154 present in circulation.[46] The levels of soluble CD154 are elevated in

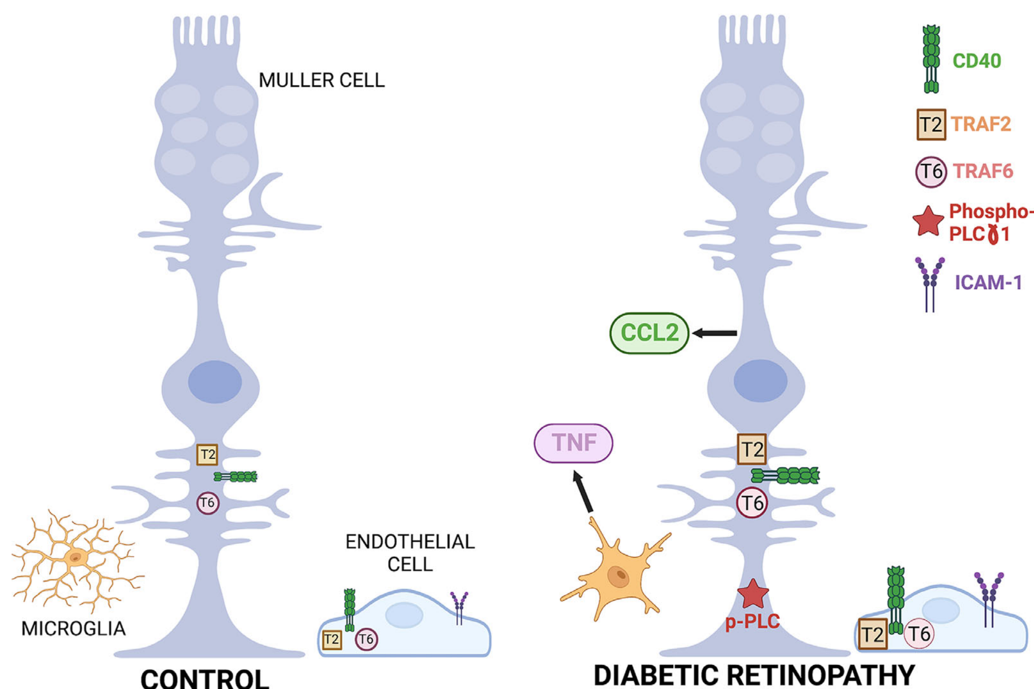

Figure 7. Model of the changes in expression of CD40, TRAF2, TRAF6, and inflammatory molecules in diabetic retinopathy in humans. Expression of CD40 and its downstream signaling molecules TRAF2 and TRAF6 are increased in retinal Müller cells and endothelial cells from patients with diabetic retinopathy. This is accompanied by increased expression of ICAM-1 in retinal endothelial cells, upregulation of CCL2 in Müller cells, and expression of activated PLCγ1 in Müller cells. TNF-α does not appear to be expressed in Müller or endothelial cells whereas it is detected in cells that likely represent microglia/macrophages. Prior in vitro studies revealed that CD40-TRAF signaling upregulates ICAM-1 in retinal endothelial cells and CCL2 production by Müller cells (Ref. 27), as well as activates PLCγ1 in Müller cells, triggering release of extracellular ATP that in turn promotes TNF-α production by myeloid cells (Ref. 28). Created with BioRender.

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the blood from patients with diabetes, particularly those with microangiopathy.[47–49] The increased levels of circulating plasma CD154 positively correlated with the presence of diabetic retinopathy and the severity of disease.[50] Importantly, CD154 upregulation is of functional relevance because serum CD154 from patients with diabetes induces pro-inflammatory responses in endothelial cells and monocytes.[49] A local increase in CD154 expression may also take place in the retina of diabetics. Infiltrating T cells in the retina and vitreous, including activated $CD4^+$ T cells, have been reported in the retinas of patients with diabetic retinopathy.[51–53] Finally, the microthrombosis that occurs in diabetic retinopathy may also contribute to increased retinal levels of CD154 given that activated platelets express this molecule.[54]

The expression of TRAFs is modulated during CD40-TRAF signaling. Although CD40 ligation can cause initial TRAF degradation,[55] studies performed 4 to 16 hours after addition of CD154 showed that CD40 ligation upregulates TRAFs.[35] Furthermore, the expression of TRAF2 and TRAF6 is increased in vivo in CD40-driven inflammatory disorders, including atherosclerosis, arterial injury, inflammatory bowel disease, and lupus nephritis.[34,35,56–59] Studies in a mouse model of arterial injury revealed that TRAF upregulation was dependent on the presence of CD40.[57] Thus, the observation that retinal cells from patients with diabetic retinopathy not only upregulate CD40 but also TRAF2 and TRAF6 suggests that CD40-TRAF signaling is activated in this disease in humans. Finally, given that TRAF2 and TRAF6 signaling mediate CD40-induced pro-inflammatory molecule upregulation in retinal cells,[27] the co-expression of CD40 with pro-inflammatory molecules raises the possibility that CD40-TRAF promotes pro-inflammatory molecule expression in patients with diabetic retinopathy. Of potential relevance, increased expression of TRAF2 correlated with the risk for relapse in patients with inflammatory bowel disease, a CD40-driven disorder.[58]

In summary, we report increased retinal expression of CD40 and its downstream signaling molecules TRAF2 and TRAF6 in patients with diabetic retinopathy as well as co-expression of CD40 with pro-inflammatory molecules key to the development of diabetic retinopathy. These findings are important because they may have therapeutic implications. Inhibition of CD40-TRAF signaling has been studied as a novel approach to control CD40-diven inflammatory disorders.[60,61] The upregulation of CD40 and TRAF2 in different retinal cell types and the key role of CD40-TRAF2 signaling as a driver of pro-inflammatory responses in numerous types of cells would support that blocking this pathway would effectively reduce retinal inflammation in diabetes. Indeed, intravitreal administration of a cell-permeable CD40-TRAF2,3 blocking peptide to mice with diabetic retinopathy ablated upregulation of a broad range of pro-inflammatory molecules as well as recruitment of leukocytes to the retina.[30] Our study raises the possibility that pharmacologic inhibition of CD40-TRAF2,3 signaling may become a novel approach to treat diabetic retinopathy in humans. Given that retinal pigment epithelial cells have basal expression of CD40,[62] future studies should examine whether CD40 expression is also increased in these cells in the diabetic retina. In addition, the in vivo role of CD40 in VEGF upregulation should be examined because both Müller cells and retinal pigment epithelial cells are important sources of VEGF in the diabetic retina.

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References

1. Cho NH, Shaw JE, Karuranga S, et al. IDF diabetes atlas: global estimates of diabetes prevalence for 2017 and projections for 2045. *Diabetes Res Clin Practice*. 2018;138:271–281.
2. Yau JW, Rogers SL, Kawasaki R, et al. Global prevalence and major risk factors of diabetic retinopathy. *Diabetes Care*. 2012;35:556–564.
3. Bourne RR, Stevens GA, White RA, et al. Causes of vision loss worldwide, 1990-2010: a systematic analysis. *Lancet Glob Health*. 2013;1:e339–e349.
4. Ciulla TA, Amador AG, Zinman B. Diabetic retinopathy and diabetic macular edema: pathophysiology, screening, and novel therapies. *Diabetes Care*. 2003;26:2653–2664.
5. Wirostko B, Wong TY, Simo R. Vascular endothelial growth factor and diabetic complications. *Prog Retin Eye Res*. 2008;27:608–621.
6. Tang J, Kern TS. Inflammation in diabetic retinopathy. *Prog Retin Eye Res*. 2011;30:343–358.
7. Antonetti DA, Klein R, Gardner TW. Diabetic retinopathy. *N Engl J Med*. 2012;366:1227–1239.
8. Rübsam A, Parikh S, Fort PE. Role of Inflammation in diabetic retinopathy. *Int J Mol Sci*. 2018;19:942.
9. McLeod DS, Lefer DJ, Merges C, Lutty GA. Enhanced expression of intracellular adhesion molecule-1 and P-selectin in the diabetic human retina and choroid. *Am J Pathol*. 1995;147:642–653.
10. Limb GA, Chignell AH, Green W, LeRoy F, Dumonde DC. Distribution of TNF alpha and its reactive vascular adhesion molecules in fibrovascular membranes of proliferative diabetic retinopathy. *Br J Ophthalmol*. 1996;80:168–173.
11. Taghavi Y, Hassanshahi G, Kounis NG, Koniari I, Khorramdelazad H. Monocyte chemoattractant protein-1 (MCP-1/CCL2) in diabetic retinopathy: latest evidence and clinical considerations. *J Cell Commun Signal*. 2019; 13:451–562.
12. Adamiec-Mroczek J, Oficjalska-Mlynczak J, Misiuk-Hojlo M. Proliferative diabetic retinopathy – The influence of diabetes control on the activation of the intraocular molecule system. *Diabetes Res Clin Practice*. 2009;84:46–50.
13. Tashimo A, Mitamura Y, Nagai S, et al. Aqueous levels of macrophage migration inhibitory factor and monocyte chemotactic protein-1 in patients with diabetic retinopathy. *Diabetic Med*. 2004;21:1292–1297.
14. Demircan N, Safran BG, Soylu M, Ozcan AA, Sizmaz S. Determination of vitreous interleukin-1 (IL-1) and tumour necrosis factor (TNF) levels in proliferative diabetic retinopathy. *Eye (Lond)*. 2006;20:1366–1369.
15. Joussen AM, Poulaki V, Le ML, et al. A central role for inflammation in the pathogenesis of diabetic retinopathy. *FASEB J*. 2004;18:1450–1452.
16. Yang L-P, Sun H-L, Wu L-M, et al. Baicalein reduces inflammatory process in a rodent model of diabetic retinopathy. *Invest Ophthalmol Vis Sci*. 2009;50:2319–2327.
17. Joussen AM, Doehmen S, Le ML, et al. TNF-α mediated apoptosis plays an important role in the development of early diabetic retinopathy and long-term histopathological alterations. *Mol Vision*. 2009;15:1418–1428.

18. Joussen AM, Poulaki V, Mitsiades N, et al. Nonsteroidal anti-inflammatory drugs prevent early diabetic retinopathy via TNF-α suppression. *FASEB J*. 2002;16:438–440.
19. Rangasamy S, McGuire PG, Franco Nitta C, Monickaraj F, Oruganti SR, Das A. Chemokine mediated monocyte trafficking into the retina: role of inflammation in alteration of the blood-retinal barrier in diabetic retinopathy. *PLoS One*. 2014;9:e108508.
20. Monickaraj F, Oruganti SR, McGuire P, Das A. A potential novel therapeutic target in diabetic retinopathy: a chemokine receptor (CCR2/CCR5) inhibitor reduces retinal vascular leakage in an animal model. *Graefes Arch Clin Exp Ophthalmol*. 2021;259:93–100.
21. Bunch KL, Abdelrahman AA, Caldwell RB, Caldwell RW. Novel therapeutics for diabetic retinopathy and diabetic macular edema: a pathophysiologic perspective. *Front Physiol*. 2022;13:831616.
22. Duh EJ, Sun JK, Stitt AW. Diabetic retinopathy: current understanding, mechanisms, and treatment strategies. *JCI Insight*. 2017;2:e93751.
23. Brown DM, Nguyen QD, Marcus DM, et al. Long-term outcomes of ranibizumab therapy for diabetic macular edema: the 36-month results from two phase III trials: RISE and RIDE. *Ophthalmology*. 2013;120:2013–2022.
24. Reddy SV, Husain D. Panretinal photocoagulation: a review of complications. *Semin Ophthalmol*. 2018;33: 83–88.
25. Boyer DS, Yoon YH, Belfort R, et al. Three-year, randomized, sham-controlled trial of dexamethasone intravitreal implant in patients with diabetic macular edema. *Ophthalmology*. 2014;121:1904–1914.
26. Portillo J-AC, Greene JA, Okenka G, et al. CD40 promotes the development of early diabetic retinopathy. *Diabetologia*. 2014;57:2222–2231.
27. Portillo J-A, Schwartz I, Zarini S, et al. Pro-inflammatory responses induced by CD40 in retinal endothelial and Muller cells are inhibited by blocking CD40-TRAF2,3 or CD40-TRAF6 signaling. *Invest Ophthalmol Vis Sci*. 2014;55:8590–8597.
28. Portillo J-AC, Lopez Corcino Y, Miao Y, et al. CD40 in retinal Muller cells induces P2X7-dependent cytokine expression in macrophages/microglia in diabetic mice and development of early experimental diabetic retinopathy in mice. *Diabetes*. 2017;66:483–493.
29. Yu JS, Daw J, Portillo JC, Subauste CS. CD40 expressed in endothelial cells promotes upregulation of ICAM-1 but not pro-inflammatory cytokines, NOS2 and P2X7 in the diabetic retina. *Invest Ophthalmol Vis Sci*. 2021;62:22.
30. Portillo JC, Yu JS, Vos S, et al. Disruption of retinal inflammation and the development of diabetic retinopathy in mice by a CD40-derived peptide or mutation of CD40 in Muller cells. *Diabetologia*. 2022;65:2157–2171.
31. Bishop GA, Moore CR, Xie P, Stunz LL, Kraus ZJ. TRAF proteins in CD40 signaling. *Adv Exp Med Biol*. 2007;597: 131–151.
32. Karmann K, Hughes CCW, Schechner J, Fanslow WC, Pober JS. CD40 on human endothelial cells: inducibility by cytokines and functional regulation of adhesion molecule expression. *Proc Natl Acad Sci USA*. 1995;92:4342–4346.
33. Bruemmer D, Riggers U, Holzmeister J, et al. Expression of CD40 in vascular smooth muscle cells and macrophages is associated with early development of human atherosclerotic lesions. *Am J Cardiol*. 2001;87:21–27.
34. Yellin MJ, D'Agati V, Parkinson G, et al. Immunohistologic analysis of renal CD40 and CD40L expression in lupus nephritis and other glomerulonephritis. *Arthritis Rheum*. 1997;40:124–134.
35. Zirlik A, Bavendiek U, Libby P, et al. TRAF-1, -2, -3, -5, and -6 are induced in atherosclerotic plaques and differentially mediate proinflammatory functions of CD40L in endothelial cells. *Atheroscler Thromb Vasc Biol*. 2007;27:1101–1107.
36. Portillo J-AC, Okenka G, Kern TS, Subauste CS. Identification of primary retinal cells and ex vivo identification of pro-inflammatory molecules in retinal cells using flow cytometry. *Mol Vis*. 2009;15:1383–1389.
37. Tan J, Town T, Mori T, et al. CD40 is expressed and functional on neuronal cells. *EMBO J*. 2002;21:643–652.
38. Kuo HL, Huang CC, Lin TY, Lin CY. IL-17 and CD40 ligand synergistically stimulate the chronicity of diabetic nephropathy. *Nephrol Dialysis Transplant*. 2018;33:248–256.
39. Chatzigeorgiou AE, Lembessis PE, Mylona-Karagianni CF, Tsouvalas EA, Diamanti-Kandarakis E, Kamper EF. CD40 expression and its association with low-grade inflammation in a Greek population of type 1 diabetic juveniles: evidence for differences in CD40 mRNA isoforms expressed by peripheral blood mononuclear cells. *Exp Clin Endocrinol Diabetes*. 2010;118:38–46.
40. Mach F, Schonbeck U, Sukhova GK, Atkinson E, Libby P. Reduction of atherosclerosis in mice by inhibition of CD40 signaling. *Nature*. 1998;394:200–203.
41. Battaglia E, Biancone L, Resegotti A, Emanuelli G, Ruggero Fronda G, Camussi G. Expression of CD40 and its ligand, CD40L, in intestinal lesions of Crohn's disease. *Am J Gastroenterol*. 1999;94:3279–3284.
42. Hakkinen T, Karkola K, Yla-Herttualla S. Macrophages, smooth muscle cells, endothelial cells, and T-cells express CD40 and CD40L in fatty streaks and more advanced human atherosclerotic lesions. *Virch Archiv*. 2000;437:396–405.
43. Ohashi Y, Okazaki H, Sato T, Miura S, Amada N, Yamaguchi M. Function of CD40/CD40L in peritubular capillary renal allograft rejection. *Transplant Proc*. 2001;33:407–408.
44. Borcherding F, Nitschke M, von Smolinski D, et al. The CD40-CD40L pathway contributes to the proinflammatory function of intestinal epithelial cells in inflammatory bowel disease. *Am J Pathol*. 2010;176:1816–1827.
45. Song Z, Jin R, Yu S, Nanda A, Granger DL, Li G. Crucial role of CD40 signaling in vascular wall cells in neointima formation and vascular remodeling after vascular interventions. *Atheroscler Thromb Vasc Biol*. 2012;32:50–64.
46. Henn V, Steinbach S, Buchner K, Presek P, Kroczek RA. The inflammatory action of CD40 ligand (CD154) expressed on activated human platelets is temporally limited by coexpressed CD40. *Blood*. 2001;98:1047–1053.
47. Varo N, Vincent D, Libby P, et al. Elevated plasma levels of the atherogenic mediator soluble CD40 ligand in diabetic patients. A novel target of thiazolidinediones. *Circulation*. 2003;107:2644–2649.
48. Yngen M, Ostenson C-G, Hu HM, Li N, Hjemdahl P, Wallen NH. Enhanced P-selectin expression and increased soluble CD40 ligand in patients with type 1 diabetes mellitus and microangiopathy: evidence for platelet hyperactivity and chronic inflammation. *Diabetologia*. 2004;47:537–540.
49. Cipollone F, Chiarelli F, Davi G, et al. Enhanced soluble CD40 ligand contributes to endothelial cell dysfunction in vitro and monocyte activation in patients with diabetes mellitus: effect of improved metabolic control. *Diabetologia*. 2005;48:1216–1224.
50. Lamine LB, Turki A, Al-Khateeb G, et al. Elevation in circulating soluble CD40 ligand concentrations in type 2 diabetic retinopathy and association with its severity. *Exp Clin Endocrinol Diabetes*. 2020;128:319–324.
51. Tang S, Le-Ruppert KC. Activated T lymphocytes in epiretinal membranes from eyes of patients with proliferative diabetic retinopathy. *Graefes Arch Clin Exp Ophthalmol*. 1995;233:21–25.
52. Kase S, Saito W, Ohno S, Ishida S. Proliferative diabetic retinopathy with lymphocyte-rich epiretinal membrane

associated with poor visual prognosis. *Invest Ophthalmol Vis Sci*. 2009;50:5909–5912.

53. Urbancic M, Kloboves Prevodnik V, Petrovic D, Globocnik Petrovic M. A flow cytometric analysis of vitreous inflammatory cells in patients with proliferative diabetic retinopathy. *Biomed Res Int*. 2013;2013:251528.
54. Boeri D, Maiello M, Lorenzi M. Increased prevalence of microthromboses in retinal capillaries of diabetic individuals. *Diabetes*. 2001;50:1432–1439.
55. Bishop GA, Hostager BS, Brown KD. Mechanisms of TNF receptor-associated factor (TRAF) regulation in B lymphocytes. *J Leuk Biol*. 2002;72:19–23.
56. Zhu L, Yang X, Ji Y, et al. Up-regulated renal expression of TNF-alpha signalling adapter proteins in lupus glomerulonephritis. *Lupus* 2009;18:116–127.
57. Song Z, Jin R, Yu S, et al. CD40 is essential in the upregulation of TRAF proteins and NF-kB-dependent proinflammatory gene expression after arterial injury. *PLoS One*. 2011;6:e23239.
58. Shen J, Qiao Y, Ran Z, Wang T, Xu J, Feng J. Intestinal protein expression profile identifies inflammatory bowel disease and predicts relapse. *Int J Clin Exp Pathol*. 2013;6:917–925.
59. Shen J, Qiao Y, Ran Z, Wang T. Different activation of TRAF4 and TRAF6 in inflammatory bowel disease. *Mediators Inflamm*. 2013;2013:647936.
60. Chatzigeorgiou A, Seijkens T, Zarzycka B, et al. Blocking CD40-TRAF6 signaling is a therapeutic target in obesity-associated insulin resistance. *Proc Natl Acad Sci USA*. 2014;111:2686–2691.
61. Portillo J-AC, Yu J-S, Hansen S, Kern TS, Subauste MC, Subauste CS. A cell-penetrating CD40-TRAF2,3 blocking peptide diminishes inflammation and neuronal loss after ischemia/reperfusion. *FASEB J*. 2021;35:e21412.
62. Van Grol J, Muniz-Feliciano L, Portillo J-AC, Bonilha VL, Subauste CS. CD40 induces anti-Toxoplasma gondii activity in non-hematopoietic cells dependent on autophagy proteins. *Infect Immun*. 2013;81:2002–2011.

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