

Proinflammatory Responses Induced by CD40 in Retinal Endothelial and Müller Cells are Inhibited by Blocking CD40-Traf2,3 or CD40-Traf6 Signaling

Jose-Andres C. Portillo,¹ Isaac Schwartz,¹ Simona Zarini,² Reena Bapputty,³ Timothy S. Kern,⁴⁻⁶ Rose A. Gubitosi-Klug,³ Robert C. Murphy,² M. Cecilia Subauste,^{5,7} and Carlos S. Subauste^{1,6,8}

¹Division of Infectious Diseases and HIV Medicine, Department of Medicine, Case Western Reserve University, Cleveland, Ohio, United States

²Department of Pharmacology, University of Colorado Denver, Aurora, Colorado, United States

³Department of Pediatrics, Case Western Reserve University/Rainbow Babies and Children's Hospital, Cleveland, Ohio, United States

⁴Division of Clinical and Molecular Endocrinology, Department of Medicine, Case Western Reserve University, Cleveland, Ohio, United States

⁵Veterans Administration Medical Center, Research Service 151, Cleveland, Ohio, United States

⁶Department of Ophthalmology and Visual Sciences, Case Western Reserve University, Cleveland, Ohio, United States

⁷Division of Pulmonary, Critical Care and Sleep Medicine, Department of Medicine, Case Western Reserve University, Cleveland, Ohio, United States

⁸Department of Pathology, Case Western Reserve University, Cleveland, Ohio, United States

Correspondence: Carlos Subauste, Division of Infectious Diseases and HIV Medicine, Department of Medicine, Case Western Reserve University, Cleveland, OH 44106, USA; carlos.subauste@case.edu.

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PURPOSE. The cell surface receptor CD40 is required for the development of retinopathies induced by diabetes and ischemia/reperfusion. The purpose of this study was to identify signaling pathways by which CD40 triggers proinflammatory responses in retinal cells, since this may lead to pharmacologic targeting of these pathways as novel therapy against retinopathies.

METHODS. Retinal endothelial and Müller cells were transduced with vectors that encode wild-type CD40 or CD40 with mutations in sites that recruit TNF receptor associated factors (TRAF): TRAF2,3 (Δ T2,3), TRAF6 (Δ T6), or TRAF2,3 plus TRAF6 (Δ T2,3,6). Cells also were incubated with CD40-TRAF2,3 or CD40-TRAF6 blocking peptides. We assessed intercellular adhesion molecule-1 (ICAM-1), CD40, monocyte chemoattractant protein-1 (MCP-1), VEGF and prostaglandin E₂ (PGE₂) by fluorescence-activated cell sorting (FACS), ELISA, or mass spectrometry. Mice (B6 and CD40^{-/-}) were made diabetic using streptozotocin. The MCP-1 mRNA was assessed by real-time PCR.

RESULTS. The CD40-mediated ICAM-1 upregulation in endothelial and Müller cells was markedly inhibited by expression of CD40 Δ T2,3 or CD40 Δ T6. The CD40 was required for MCP-1 mRNA upregulation in the retina of diabetic mice. The CD40 stimulation of endothelial and Müller cells enhanced MCP-1 production that was markedly diminished by CD40 Δ T2,3 or CD40 Δ T6. Similar results were obtained in cells incubated with CD40-TRAF2,3 or CD40-TRAF6 blocking peptides. The CD40 ligation upregulated PGE₂ and VEGF production by Müller cells, that was inhibited by CD40 Δ T2,3 or CD40 Δ T6. All cellular responses tested were obliterated by expression of CD40 Δ T2,3,6.

CONCLUSIONS. Blockade of a single CD40-TRAF pathway was sufficient to impair ICAM-1, MCP-1, PGE₂, and VEGF upregulation in retinal endothelial and/or Müller cells. Blockade of CD40-TRAF signaling may control retinopathies.

Keywords: diabetes, CD40, intercellular adhesion molecules, chemokine

The cell surface receptor CD40 is a receptor constitutively expressed on antigen-presenting cells that also can be present on nonhematopoietic cells.¹ Its counter-receptor, CD154 (CD40 ligand) is expressed on activated CD4⁺ T cells and platelets.¹ The receptor CD154 also exists as a biologically active soluble protein present in plasma.² The CD40-CD154 interaction promotes the development of various inflammatory and autoimmune disorders.^{1,3}

Nonhematopoietic cells are either CD40⁻ or express low levels of CD40 under basal conditions. However, CD40 is

induced or upregulated in these cells during inflammation.⁴⁻⁶ We reported that retinal endothelial cells, Müller cells, ganglion neurons, retinal microglia, and RPE cells express CD40 at low levels (corrected mean fluorescence intensity between 100 and 160).⁷⁻¹⁰ Moreover, retinal CD40 mRNA expression increases in diabetic mice and in mice subjected to retinal ischemia/reperfusion^{7,9} (and Portillo et al., unpublished observations, 2008). In the case of diabetes, CD40 upregulation occurs in retinal endothelial cells, Müller cells, and microglia.⁹ Study of the regulation of CD40-mediated proinflammatory responses in

retinal cells is important because of the pathogenic role of CD40 in retinopathies with an inflammatory component. Indeed, CD40^{-/-} mice are protected from ischemia/reperfusion-induced retinopathy and early diabetic retinopathy.^{7,9}

The role of the CD40-CD154 pathway in various disorders with an inflammatory component made it an attractive therapeutic target. Administration of blocking anti-CD154 mAb showed therapeutic efficacy in mice.¹¹ However, clinical trials of anti-CD154 mAb administration for Crohn's disease, lupus nephritis, and idiopathic thrombocytopenic purpura were stopped due to thromboembolic events.¹² Since CD40 is the major receptor for CD154, blockade of signaling downstream of CD40 may represent an alternative approach to inhibit the CD40-CD154 pathway. Therapeutic strategies to block this pathway must take into account that CD40-CD154 signaling also is central for protection against a broad variety of pathogens.¹ Thus, the approaches to inhibit CD40 signaling ideally should be selective enough to impair proinflammatory responses while minimizing the risk of immunosuppression.

The CD40 receptor signals via adaptor proteins, such as TNF receptor-associated factors (TRAF) and JAK3.^{13,14} The TRAF factors are key mediators of CD40 signaling.¹³ Receptor CD40 has domains that directly bind TRAF2 and TRAF3^{15,16} (TRAF3 typically inhibits CD40 signaling), and a domain that binds TRAF6.¹⁵ Although there can be overlap in the cellular responses induced by the TRAF2,3 and TRAF6 binding sites, responses triggered by these sites can be distinct. The TRAF6 binding site drives IL-12 secretion by dendritic cells, dendritic cell maturation, TNF- α , IL-1 β , IL-6, and nitric oxide synthase 2 (NOS2) upregulation in macrophages, autophagy-mediated antimicrobial activity in macrophages, IL-6 production by B cells, and plasma cell formation.¹⁷⁻²⁴ On the other hand, the TRAF2,3 binding site promotes immunoglobulin isotype switch.²⁵

The role of CD40-TRAF signaling in retinal cells is unknown. We generated retroviral vectors that encode wt CD40 or CD40 with mutations in the TRAF2,3 (Δ T2,3), TRAF6 (Δ T6), or TRAF2,3 plus TRAF6 binding sites (Δ T2,3,6) reported to prevent recruitment of the appropriate TRAFs.²⁶⁻²⁸ This approach is well suited to studying TRAF signaling downstream of CD40.^{18,25,28,29} Using human retinal endothelial cells, Müller cells, and a rat Müller cell line transduced with these retroviral vectors, we examined the role of TRAF binding sites in the upregulation of intercellular adhesion molecule-1 (ICAM-1), monocyte chemoattractant protein-1 (MCP-1), VEGF and prostaglandin E₂ (PGE₂). These molecules were studied since they are upregulated after retinal ischemia-reperfusion and/or in the diabetic retina, and are considered to have a pathogenic role in these retinopathies.³⁰⁻³⁵

MATERIALS AND METHODS

Cells

Primary human retinal endothelial and Müller cells were obtained as described.⁹ Endothelial cells were cultured in gelatin-coated tissue culture flasks containing Dulbecco's modified Eagle medium (DMEM) plus 10% fetal bovine serum (FBS; HyClone, Logan, UT, USA) supplemented with endothelial cell growth supplement from bovine pituitary (15 μ g/mL; Sigma-Aldrich, St. Louis, MO, USA) and insulin/transferrin/selenium (Sigma-Aldrich). Cell identity was confirmed by incorporation of acetylated low-density lipoprotein (>90%). Human Müller cells were cultured in DMEM/F12 containing 20% FBS. Cultures were >95% pure for Müller cells (vimentin⁺, CRALBP⁺, and GFAP⁻ by immunofluorescence). Human retinal cells were used between passages 3 to 6. The rat Müller cell

line rMC-1 was a gift from Dr V. R. Sarthy (Northwestern University, Chicago, IL, USA).³⁶

In Vitro Stimulation

To induce CD40 stimulation, cells were treated with multimeric human CD154 (gift from Richard Kornbluth, MD, PhD, Multimeric Biotherapeutics, Inc., La Jolla, CA, USA) for 24 hours as described.⁷ Specificity was confirmed by detecting >95% neutralization of the responses after addition of anti-human CD154 mAb (Ancell Corporation, Bayport, MN, USA). As controls we used omission of CD154 or incubation with a nonfunctional CD154 mutant (T147N).³⁷ Endothelial cells also were incubated with IFN- γ (500 IU/mL; PeproTech, Rocky Hill, NJ, USA) plus TNF- α (500 IU/mL; PeproTech). In some experiments Celecoxib (1 μ M; Sigma-Aldrich) was added to cells 1 hour before stimulation with CD154.

Retroviral Vectors and Transductions

The cDNA for wild-type human CD40, CD40 Δ 22 (a mutant that ablates binding to TRAF2 and TRAF3; Δ TRAF2, 3), CD40EEAA (a mutant that prevents binding to TRAF6; Δ TRAF6), and CD40 Δ 55 (a mutant that ablates binding to TRAF2, TRAF3, and TRAF6; Δ TRAF2, 3, 6) were gifts from Gail Bishop, PhD (University of Iowa, Iowa City, IA, USA).^{28,38} The CD40 cDNA were cloned into the murine stem cell virus-based bicistronic retroviral vector MIEG3 that encodes enhanced GFP (EGFP)³⁹ as described previously.⁴⁰ Ecotropic retroviral supernatants were generated by transfecting the Phoenix-gp cell line with MIEG3-based retroviral vectors and plasmids encoding envelop (RD114; gift from Yasu Takeuchi, University College London, London, UK) and gag-pol using the calcium phosphate transfection kit (Invitrogen Life Technologies, Carlsbad, CA, USA) as described.⁴⁰ Cells were incubated overnight with retrovirus in the presence of polybrene (8 μ g/mL; Sigma-Aldrich). Cells were washed and used at least 48 hours after infection with retroviruses.

Cell-Permeable Peptides

Peptides consisted of the amino acid sequence of the TRAF2,3 or TRAF6 binding site of CD40 followed by the TAT₄₇₋₅₇ cell penetrating peptide. The sequences for the CD40-TRAF2,3 and the CD40-TRAF6 blocking peptides were NH₂-NTAAPV QETLHG YGRKKRRQRRR-OH and NH₂-KQEPQEIDFPDD YGRKKRRQRRR-OH. The TAT₄₇₋₅₇ sequence is underlined. Control peptides were TAT₄₇₋₅₇ or TAT₄₇₋₅₇ linked to scrambled peptide. No differences in the effects between control peptides were noted. Peptides were manufactured by Proteintech Group (San Diego, CA, USA) and were low in endotoxin and >98% pure by HPLC. Peptides were added to cells 3 hours before stimulation with CD154.

Flow Cytometry

Retinal cells were incubated with human IgG (Jackson ImmunoResearch Laboratories, West Grove, PA, USA) followed by staining with anti-human CD40 PE (BD Biosciences, San Jose, CA, USA), anti-human ICAM-1 PE (eBiosciences, San Diego, CA, USA), anti-rat ICAM-1 PerCP-eFluor 710 (eBiosciences), or appropriate isotype control mAbs. After fixation with 1% paraformaldehyde, flow cytometry data acquisition was performed using a LSR II and running FACSDiva software (Becton Dickinson, San Jose, CA, USA). FlowJo software (Tree Star, Inc., Ashland, OR, USA) was used for data analysis. Expression of CD40 and ICAM-1 is expressed as corrected mean fluorescence intensity (cMFI).

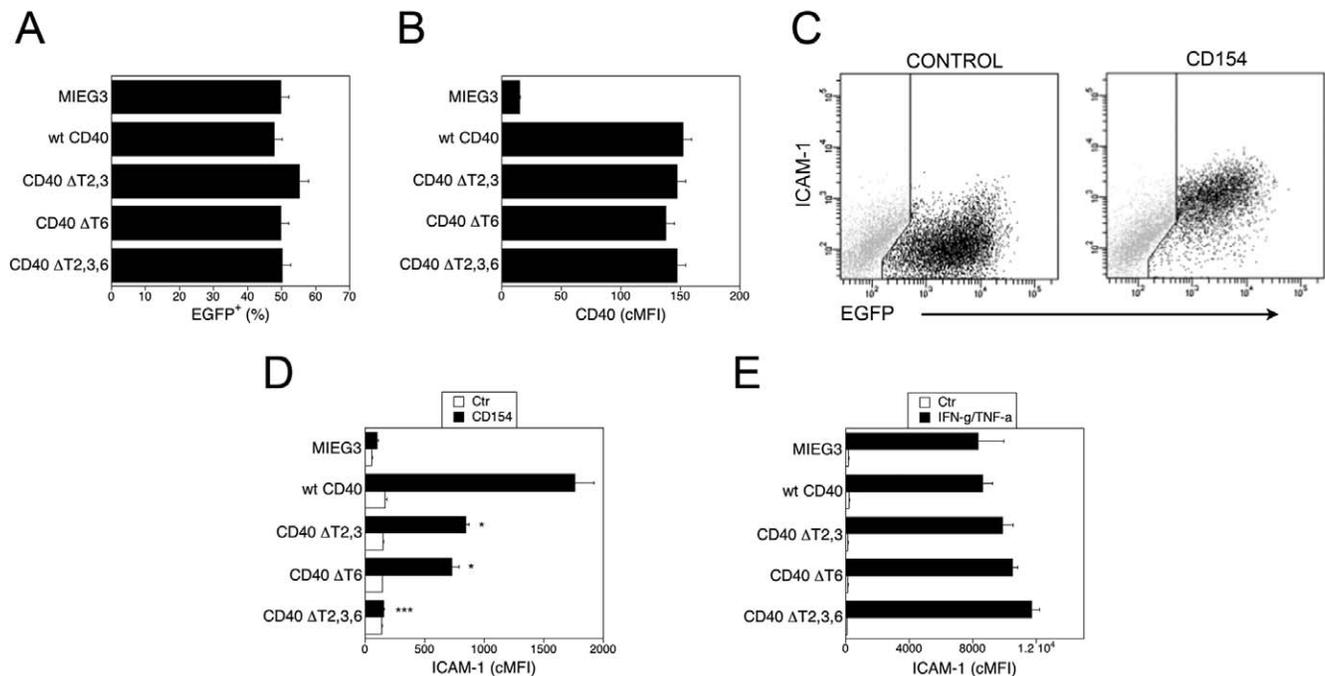


FIGURE 1. Role of CD40-TRAF binding sites on ICAM-1 upregulation in HREC. The HREC were transduced with MIEG3-based retroviral vector that encode EGFP and either wt CD40, CD40 Δ T2,3, CD40 Δ T6, CD40 Δ T2,3,6. (A) Percentages of HREC that became EGFP⁺ after incubation with retroviral vectors. (B) Expression of CD40 on gated EGFP⁺ cells shown as corrected mean fluorescence intensity (cMFI). (C) Dot plots of HREC transduced with wt CD40-encoding retroviral vector depicting expression of ICAM-1 and EGFP at 24 hours post-incubation with or without CD154. (D) The HREC transduced with the retroviral vectors were incubated with or without CD154 and expression of ICAM-1 (cMFI) on gated EGFP⁺ cells was assessed by flow cytometry at 24 hours. (E) The HREC transduced with the retroviral vectors were incubated with IFN- γ plus TNF- α and expression of ICAM-1 on gated EGFP⁺ cells was assessed by flow cytometry at 24 hours. Results are shown as mean \pm SEM and are representative of 3 to 4 experiments. * P < 0.05, *** P < 0.001 represent comparison to cells that express wt CD40.

Cytokine ELISA

Cell-free supernatants were collected at 24 hours and used to measure concentrations of MCP-1 and VEGF (R & D Systems, Minneapolis, MN, USA).

Measurement of PGE2

Culture supernatants were subjected to solid phase extraction.⁴¹ The PGE₂ concentrations were analyzed by reverse-phase HPLC and electrospray ionization mass spectrometry as previously described.⁴¹ The PGE₂ also was measured by ELISA (Cayman Chemical, Ann Arbor, MI, USA).

Statistical Analysis

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

RESULTS

Role of the CD40-TRAF2,3 and the CD40-TRAF6 Binding Sites in CD154-Induced Upregulation of ICAM-1 in Human Retinal Endothelial Cells (HRECs)

Primary HRECs were transduced with bicistronic retroviral vectors that encode EGFP and either wt CD40 or CD40 mutants lack binding to TRAF2,3 (Δ T2,3), TRAF6 (Δ T6), or TRAF2,3,6 (Δ T2,3,6).^{26–28} The percentages of transduced cells (EGFP⁺) and the cMFI for CD40 on EGFP⁺ cells were similar for

all vectors (Figs. 1A, 1B; P > 0.5). The CD154 markedly upregulated ICAM-1 on transduced (EGFP⁺) HREC that expressed wt CD40 (Fig. 1C). This effect was specific since it was obliterated by a neutralizing anti-CD154 mAb (>95% inhibition; data not shown). Consistent with the low CD40 expression in HREC under basal conditions, HREC transduced with the empty retroviral vector (MIEG3) exhibited less pronounced upregulation of ICAM-1 (mean cMFI Ctr = 58; CD154 = 103) in response to CD154. Thus, cellular responses in transduced HAEC are driven mainly by retroviral-induced CD40. Expression of either CD40 Δ T2,3 or CD40 Δ T6 markedly inhibited ICAM-1 upregulation, while the expression of CD40 Δ T2,3,6 obliterated this response (Fig. 1D). The effects of the mutations were specific, since upregulation of ICAM-1 in response to IFN- γ /TNF- α was similar regardless of the retroviral vector used (Fig. 1E, P > 0.1). Thus, a mutation that prevents CD40-TRAF2,3 or CD40-TRAF6 interaction is sufficient to inhibit ICAM-1 upregulation in HREC.

CD40 Drives MCP-1 Upregulation in the Retina of Diabetic Mice

Before examining whether CD40-TRAF signaling in retinal cells regulates MCP-1 production, we tested whether CD40 drives retinal MCP-1 upregulation in vivo. Male B6 and CD40^{-/-} mice were rendered diabetic by administration of streptozotocin. Throughout the study B6 and CD40^{-/-} mice exhibited similar blood glucose concentrations (B6 = 364 \pm 13 mg/mL; CD40^{-/-} = 361 \pm 7 mg/mL) as well as hemoglobin A_{1c} (HbA_{1c}) levels (B6 = 8.7 \pm 0.3%; CD40^{-/-} = 8.4 \pm 0.3%; P > 0.5). Diabetic B6 mice, but not diabetic CD40^{-/-} mice, upregulated MCP-1 (Table 1).

TABLE 1. Changes in mRNA Levels of MCP-1 in the Retinas of Diabetic Mice

	B6 Control	B6 Diabetic	CD40 ^{-/-} Control	CD40 ^{-/-} Diabetic
MCP-1 mRNA, relative levels	1.0 ± 0.15	2.2 ± 0.32*	1.06 ± 0.1	1.13 ± 0.1

At 2 months of diabetes, retinas from diabetic B6 and CD40^{-/-} mice as well as from control (nondiabetic) animals were collected and used for mRNA extraction. The mRNA levels of MCP-1 were assessed by real-time quantitative PCR using 18S rRNA as internal control. Data are expressed as fold-increase in diabetic mice compared to retinas from nondiabetic controls. Data shown represent mean ± SEM (10 to 15 animals per group).

* Diabetic B6 mice showed significantly higher MCP-1 upregulation ($P < 0.05$) compared to control B6 mice and diabetic CD40^{-/-} mice.

Role of the CD40-TRAF2,3 and the CD40-TRAF6 Binding Sites in CD154-Induced Upregulation of MCP-1 in HRECs

The CD154 stimulated MCP-1 production by HREC that expressed wt CD40 (Fig. 2). The expression of CD40 Δ T2,3,6 obliterated the MCP-1 production induced by CD154 (Fig. 2). Expression of CD40 Δ T2,3 or CD40 Δ T6 also markedly inhibited MCP-1 production (Fig. 2). Thus, a mutation in either the TRAF2,3 or TRAF6 binding sites is sufficient to impair MCP-1 production by HREC.

Effects of Pharmacologic Inhibition of CD40-TRAF Signaling in CD154-Induced Upregulation of ICAM-1 in HRECs

We reported that cell permeable peptides that include the amino acid sequence of the TRAF2,3 or TRAF6 binding site of CD40 block appropriate CD40-TRAF signaling.²³ We incubated untransduced HREC with peptides that consisted of the amino acid sequence of the TRAF2,3 or the TRAF6 binding sites of CD40 linked to TAT₄₇₋₅₇. The HRECs then were stimulated with CD154. The CD40-TRAF2,3 and CD40-TRAF6 blocking peptides impaired upregulation of ICAM-1 in response to CD154 (Fig. 3). Taken together, not only genetic but also a pharmacologic approach to block CD40-TRAF2,3 or CD40-TRAF6 signaling inhibit a CD40-mediated proinflammatory response in HREC.

Role of CD40-TRAF Binding Sites in ICAM-1 and MCP-1 by Human Retinal Müller Cells (HRMCs)

The HRMCs were transduced with the retroviral vectors that encode wt or CD40 mutants. The percentages of transduced cells (EGFP⁺) and the cMFI for CD40 on transduced cells were similar for all groups (Figs. 4A, 4B; $P > 0.5$). Incubation with CD154 upregulated ICAM-1 and MCP-1 production in HRMC that expressed wt CD40 (Figs. 4C, 4D). These responses were

obliterated in HRMC that expressed CD40 Δ T2,3,6 (Fig. 5C). Expression of CD40 Δ T2,3 or CD40 Δ T6 were sufficient to markedly inhibit upregulation of ICAM-1 and MCP-1 (Figs. 4C, 4D).

We determined whether CD40-TRAF signaling also controls a proinflammatory response in rodent Müller cells. We used a rat Müller cell line transduced with retroviral vectors that encode a chimera of the extracellular domain of human CD40 and the intracellular domain of mouse CD40 (hmCD40). Addition of human CD154 to rodent cells that express this chimera results in functional CD40 signaling.²² Moreover, the TRAF binding motives are similar among various species, such as mouse, rat, humans (TRAF2,3 proximal: PxQxT; TRAF2 distal SVxE; TRAF6: PxExxAr/Ac), and CD40 can interact and signal through TRAFs of different species (for example human to mouse).^{15,16,40} Rat Müller cells (rMC1) were transduced with retroviral vectors that encode hmCD40 chimeras with mutations in the TRAF2,3, TRAF6, or TRAF2,3 plus TRAF6 binding sites.²² Transduced cell lines were >95% EGFP⁺. The levels of CD40 expression were similar for all vectors (Fig. 4E). The ICAM-1 upregulation was TRAF binding site-dependent, since it was impaired in Müller cells that expressed hmCD40 Δ T2,3 or hmCD40 Δ T6 (Fig. 4F).

The HRMC transduced with wt-CD40-encoding vector were incubated with the CD40-TRAF2,3 blocking peptides, CD40-TRAF6 blocking peptide, or control peptide followed by stimulation with CD154. The CD40-TRAF2,3 and CD40-TRAF6 blocking peptides inhibited MCP-1 production by CD154-treated HRMC (Fig. 5). Taken together, inhibition of CD40-TRAF2,3 or CD40-TRAF6 via genetic or pharmacologic approaches impair CD40-induced proinflammatory responses in Müller cells.

Role of CD40-TRAF Binding Sites in PGE2 and VEGF Production by Retinal Müller Cells

It is not known if CD40 stimulates PGE₂ and VEGF production by Müller cells. The HRMC that express wt CD40 upregulated production of PGE₂, an effect that was obliterated by the COX-2

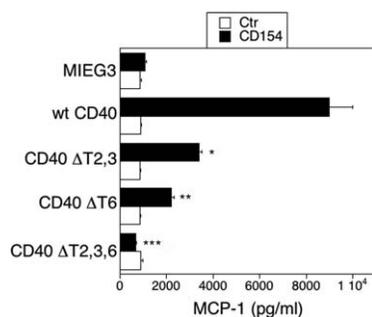


FIGURE 2. Role of CD40-TRAF binding sites on MCP-1 production in HREC. The HREC transduced with the retroviral vectors were incubated with or without CD154 for 24 hours and MCP-1 concentrations in supernatants determined by ELISA. Results are shown as mean ± SEM and are representative of 3 to 4 experiments. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ represent comparison to cells that express wt CD40.

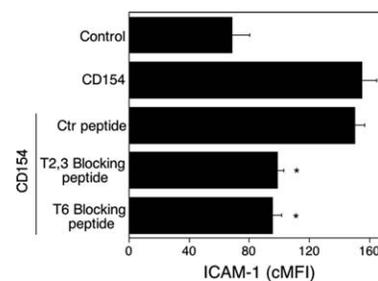


FIGURE 3. Effects of CD40-TRAF blocking peptides on ICAM-1 upregulation in HREC stimulated with CD154. Three hours before the onset of stimulation with CD154, HREC were incubated with control, CD40-TRAF2,3, or CD40-TRAF6 blocking peptides (1 μ M). Cells then were incubated with or without CD154 for 24 hours. The ICAM-1 expression was examined by flow cytometry. Results are shown as mean ± SEM and are representative of 3 experiments. * $P < 0.05$, represent comparison to cells treated with control peptide.

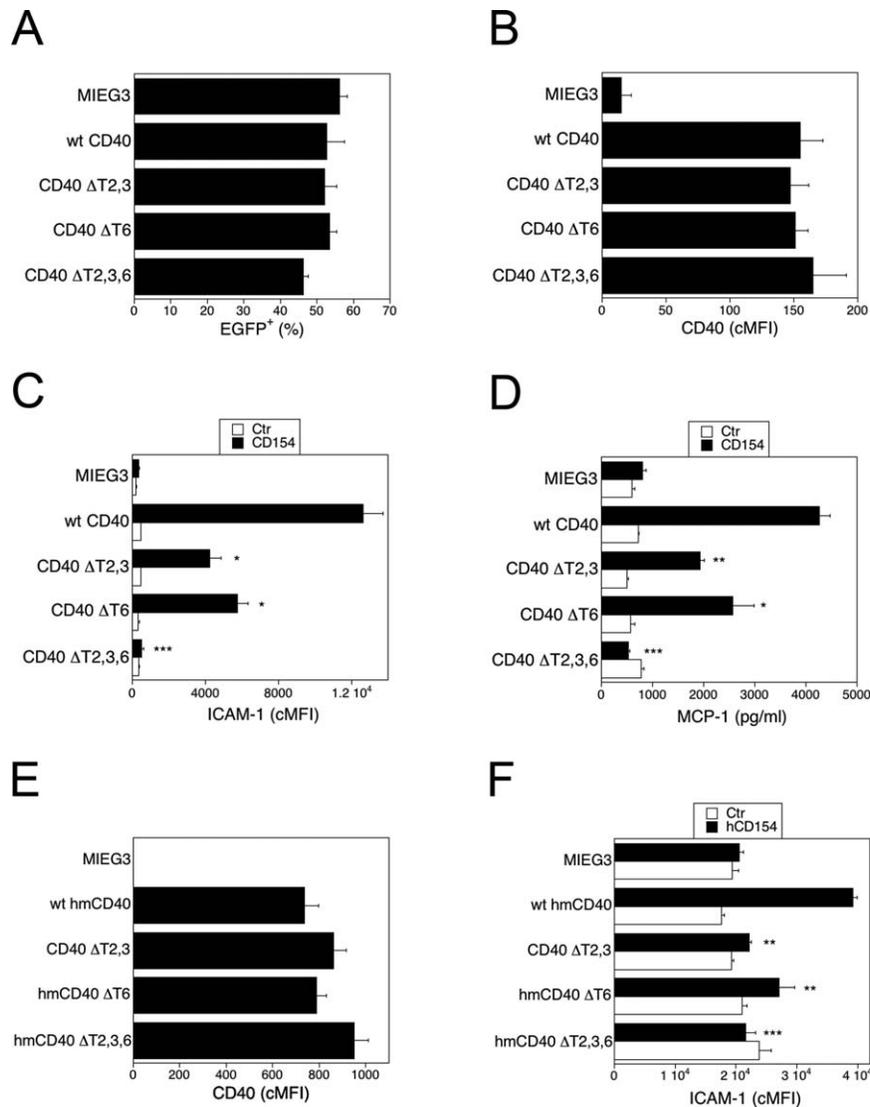


FIGURE 4. Role of CD40-TRAF binding sites on ICAM-1 upregulation and MCP-1 production in Müller cells. (A–D) The HRMC were transduced with MIEG3-based retroviral vector that encode either wt CD40, CD40 ΔT2,3, CD40 ΔT6, or CD40 ΔT2,3,6. Percentages of HRMC that became EGFP⁺ after incubation with retroviral vectors (A) and expression of CD40 (cMFI) on gated EGFP⁺ cells (B). The HRMC transduced with the retroviral vectors were incubated with or without CD154 for 24 hours. Expression of ICAM-1 (cMFI) on gated EGFP⁺ cells was assessed by flow cytometry (C) and MCP-1 concentrations in supernatants determined by ELISA (D). (E, F) Rat Müller cells (rMC1) were transduced with MIEG3-based retroviral vector that encode EGFP and either wt human-mouse CD40, human-mouse CD40 ΔT2,3, human-mouse CD40 ΔT6, or human-mouse CD40 ΔT2,3,6. Expression of CD40 (cMFI) on gated EGFP⁺ cells was assessed by flow cytometry (E). (F) Cells were incubated with or without human CD154 for 24 hours. Expression of ICAM-1 (cMFI) on gated EGFP⁺ cells was assessed by flow cytometry at 24 hours. Results are shown as mean ± SEM and are representative of 3 to 4 experiments. **P* < 0.05, ***P* < 0.01, ****P* < 0.001 represent comparison to cells that express wt CD40.

inhibitor celecoxib (Fig. 6A). The PGE₂ production was inhibited in HRMC that expressed CD40 ΔT2,3 or CD40 ΔT6 and was obliterated in those that expressed CD40 ΔT2,3,6 (Fig. 6B). Similarly, CD40 ligation caused VEGF upregulation that was dependent of the CD40-TRAF2,3 and CD40-TRAF6 binding sites (Fig. 6C). Table 2 summarizes the inhibitory effects of the mutations in the TRAF binding sites. Taken together, blockade of CD40-TRAF2,3 or CD40-TRAF6 signaling was sufficient to markedly inhibit proinflammatory responses in retinal endothelial and Müller cells.

DISCUSSION

The CD40 receptor stimulates inflammatory responses in the retina and is a major driver of early diabetic retinopathy and

ischemia/reperfusion-induced retinopathy.^{7,9} The signaling cascades through which CD40 induces proinflammatory responses in retinal cells remained unknown. Using cells that express CD40 with mutations in TRAF binding sites we report that ICAM-1 upregulation and MCP-1 production by retinal endothelial cells and Müller cells as well as PGE₂ and VEGF production by Müller cells are dependent on TRAFs. The TRAF2,3 and the TRAF6 binding sites of CD40 are required for the optimal induction of these responses. Importantly, blockade of one of these signaling pathways is sufficient to markedly inhibit ICAM-1 upregulation and production of MCP-1, PGE₂, and VEGF. We also report that CD40-TRAF blocking peptides impaired these CD40-induced proinflammatory responses. The fact that in vivo delivery of cell permeable peptides to various retinal cells occurs after intravitreal injection⁴² raises the possibility that a pharmacologic approach

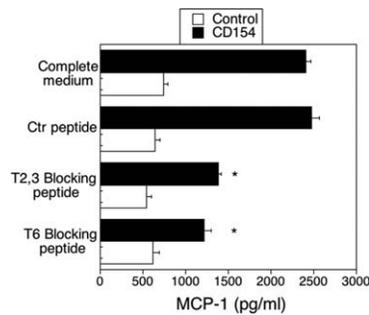


FIGURE 5. Effects of CD40-TRAF blocking peptides on MCP-1 production in HRMC stimulated with CD154. Three hours before the onset of stimulation with CD154, HRMC transduced with wt CD40-encoding retroviral vector were incubated with control, CD40-TRAF2,3 or CD40-TRAF6 blocking peptides (10 μ M). The MCP-1 concentrations in supernatants collected at 24 hours were determined by ELISA. Results are shown as mean \pm SEM and are representative of 3 experiments. * $P < 0.05$, represent comparison to cells treated with control peptide.

to prevent CD40-TRAF interaction may prove useful for the management of retinopathies driven by CD40.

Studies with bone marrow transplants indicate that leukocytes have an important role in the development of diabetic retinopathy.⁴³ However, nonhematopoietic cells also are important in the development of inflammatory disorders. Using bone marrow transplants in a mouse model of ischemia/reperfusion-induced retinopathy, we reported that absence of CD40 in the retina inhibited ICAM-1 upregulation, leukocyte recruitment to the retina and neurovascular degeneration.⁷ The CD40 is operative in both hematopoietic and non-hematopoietic cellular compartments. Indeed, studies in an animal model of arterial injury revealed that expression of CD40 at the level of leukocytes and vascular wall cells is critical for neointima formation and vascular inflammation.^{44,45}

The proinflammatory responses driven by the CD40-TRAF binding sites are likely of pathogenic relevance to retinopathies. The ICAM-1 is upregulated in the retina and in retinal endothelial cells after ischemia/reperfusion, as well as in the diabetic retina, phenomena that are dependent on CD40.⁷⁻⁹ Blockade or deficiency of ICAM-1 diminishes retinal injury following ischemia³⁰ and capillary degeneration in diabetes.³² We report that CD40 mediates the upregulation of MCP-1 in

TABLE 2. Effects of Expression of CD40 with Mutations in CD40-TRAF Binding Sites on Upregulation of Various Proinflammatory Responses on HREC, HRMC and Rat Müller Cells

	Δ TRAF2,3	Δ TRAF6	Δ TRAF2,3,6
HERCs			
ICAM-1	56.5 \pm 6.3	51.0 \pm 6.5	99.6 \pm 0.4
MCP-1	64.7 \pm 7.0	69.0 \pm 8.5	99.9 \pm 0.1
HRMCs			
ICAM-1	59.6 \pm 5.3	54.9 \pm 7.6	99.1 \pm 0.4
MCP-1	75.4 \pm 6.1	60.6 \pm 8.0	97.3 \pm 2.7
PGE ₂	83.0 \pm 5.0	91.9 \pm 7.8	97.1 \pm 2.9
VEGF	81.5 \pm 7.1*	54.6 \pm 2.3	97.4 \pm 1.6
Rat Müller cells			
ICAM-1	77.5 \pm 4.8	68.3 \pm 2.0	98.1 \pm 1.9

Data are expressed as mean inhibition compared to cells that expressed wt CD40. Results are averages \pm SEM from 3 to 4 experiments. Inhibitory effects of the Δ T2,3 and Δ T6 mutations were similar with the exception of the effects on VEGF production by Müller cells.

* $P < 0.05$.

the retina of diabetic mice, a chemokine that appears to have a pathogenic role in proliferative diabetic retinopathy in humans.³¹ The CD40-TRAF signaling enhances PGE₂ and VEGF production by Müller cells, molecules that are elevated in diabetic and ischemic retinopathies and are considered to drive microvascular complications and neovascularization that accompany these diseases.³³⁻³⁵

One of the concerns with indiscriminate inhibition of CD40 signaling is that it would cause susceptibility to various infectious diseases. The CD40-TRAF6 signaling in MHC class II⁺ cells promotes vascular inflammation in vivo.^{44,46} Indeed, blockade of this pathway has been proposed as an approach to control CD40-mediated inflammatory disorders. However, the CD40-TRAF6 pathway also drives immune responses required for protection against infections. The TRAF6 binding site of CD40 is essential for CD40-mediated production of IL-12 in dendritic cells and dendritic cell maturation.^{19,24} This binding site also is responsible for autophagy-dependent and NOS2-dependent induction of macrophage antimicrobial activity^{22,23,40} as well as production of IL-1 β , IL-6, and TNF- α .²⁰ In addition, the CD40-TRAF6 binding site also regulates humoral

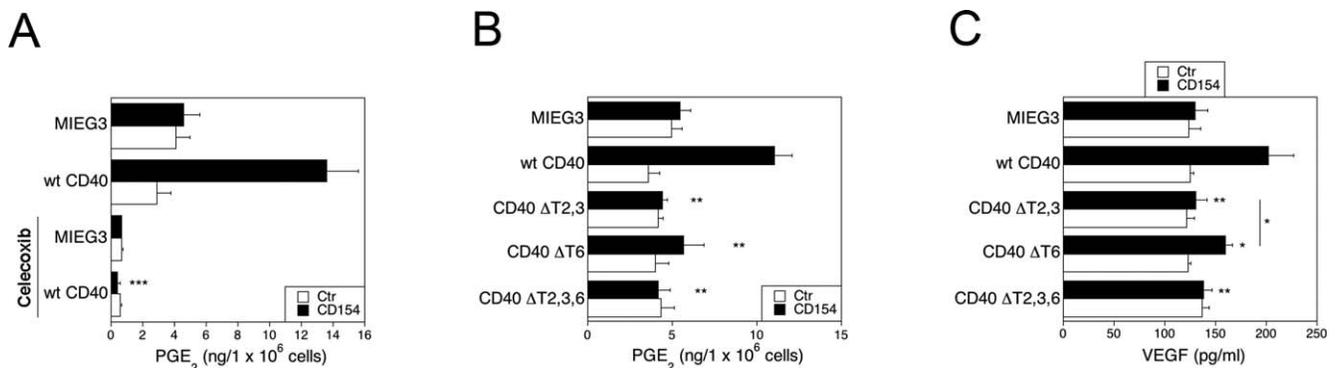


FIGURE 6. Role of CD40-TRAF binding sites on PGE₂ and VEGF production by HRMC. (A) The HRMC were transduced with MIEG3-based retroviral vector that encode EGFP with or without human wt CD40. Cells were incubated with or without CD154 for 24 hours in the presence of absence of celecoxib (1 μ M). The PGE₂ was measured by mass spectrometry or ELISA. *** $P < 0.001$ represent comparison to cells with wt CD40 that were incubated with CD154 alone. (B, C) Production of PGE₂ (B) and VEGF (C) by HRMC transduced with MIEG3-based retroviral vector that encode EGFP and either human wt CD40, CD40 Δ T2,3, CD40 Δ T6, CD40 Δ T2,3,6. Cells were incubated with or without CD154 for 24 hours. The VEGF concentrations were determined by ELISA. Results are shown as mean \pm SEM and are representative of 3 experiments. * $P < 0.05$, ** $P < 0.01$ represent comparison to cells that express wt CD40.

immunity, since it promotes affinity maturation and the generation of long lived plasma cells.¹⁸ In contrast, while the proximal CD40-TRAF2,3 binding site drives immunoglobulin isotype switch,²⁵ the TRAF2,3 binding sites do not have an appreciable role in the cellular immune responses described above.^{19,20,22,23,40} Our findings suggested that blockade of the CD40-TRAF2,3 pathway may represent an effective way to dampen CD40-mediated inflammation while likely having less inhibitory effect on CD40-mediated host protection than blockade of CD40-TRAF6 signaling. The development of CD40-TRAF2,3 inhibitors may lead to a novel therapeutic approach to control retinopathies, such as those induced by diabetes and ischemia.

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