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CD40 promotes the development of early diabetic retinopathy in mice

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Abstract

Aims/hypothesis—Microangiopathy is a leading complication of diabetes that commonly affects the retina. Degenerate capillaries are a central feature of diabetic retinopathy. An inflammatory process has been linked to the development of diabetic retinopathy but its regulation is incompletely understood. Cluster of differentiation (CD) 40 is a member of the TNF receptor superfamily that promotes the development of certain inflammatory disorders. The role of CD40 in diabetic microangiopathy is unknown.

Methods—B6 and *Cd40*$^{-/-}$ mice were administered streptozotocin to induce diabetes. Leucostasis was assessed using fluorescein isothiocyanate-conjugated concanavalin A. Retinal *Icam1* and *Cd40* mRNA levels were examined using real-time PCR. Protein nitration was assessed by immunohistochemistry. Histopathology was examined in the retinal vasculature. CD40 expression was assessed by flow cytometry and immunohistochemistry. Intercellular adhesion molecule 1 (ICAM-1) and nitric oxide synthase 2 (NOS2) were examined by immunoblot and/or flow cytometry. Nitric oxide production was examined by immunoblot and Griess reaction.

Results—In mouse models of diabetes, *Cd40*$^{-/-}$ mice exhibited reduced retinal leucostasis and did not develop capillary degeneration in comparison with B6 mice. Diabetic *Cd40*$^{-/-}$ mice had diminished ICAM-1 upregulation and decreased protein nitration. *Cd40* mRNA levels were

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increased in the retinas of diabetic B6 mice compared with non-diabetic controls. CD40 expression increased in retinal Müller cells, endothelial cells and microglia of diabetic animals. CD40 stimulation upregulated ICAM-1 in retinal endothelial cells and Müller cells. CD40 ligation upregulated NOS2 and nitric oxide production by Müller cells.

Conclusions/interpretation—CD40-deficient mice were protected from the development of diabetic retinopathy. These mice exhibited diminished inflammatory responses linked to diabetic retinopathy. CD40 stimulation of retinal cells triggered these pro-inflammatory responses.

Keywords

CD40; Diabetic retinopathy; Endothelial cells; Inflammation; Müller cells; Pathogenesis; Retina

Introduction

Diabetic retinopathy is the leading cause of vision loss among working-age adults in developed countries. Important features of early vascular lesions of diabetic retinopathy include increased death of retinal endothelial cells and pericytes [1]. The transformation of capillaries into tubes of basement membrane devoid of cells (capillary degeneration) is relevant because degenerate retinal capillaries lack blood flow and, thus, likely contribute to retinal ischaemia and subsequent neovascularisation.

Increasing evidence indicates that a chronic low-grade inflammatory response plays an important role in the pathogenesis of diabetic retinopathy [2]. The number of leucocytes adherent to vessel walls is increased in the retinal vasculature during diabetes, a phenomenon mediated by upregulation of intercellular adhesion molecule 1 (ICAM-1) [3]. Blockade of the interaction between ICAM-1 and cluster of differentiation (CD) 18 diminishes capillary degeneration [4]. Inducible nitric oxide synthase 2 (NOS2) is expressed in the retinas of patients with diabetic retinopathy and in those of rat models of diabetes [5, 6]. Moreover, diabetic *Nos2*$^{-/-}$ mice have diminished retinal leucostasis and capillary degeneration [7, 8]. While these represent significant advances in understanding the pathogenesis of diabetic retinopathy, the mechanisms that regulate these mediators of disease are not well understood. Although the pathogenesis of diabetic retinopathy is complex, discovery of an upstream molecule that controls these pro-inflammatory responses may enhance the possibility of finding effective approaches for the prevention and management of diabetic retinopathy.

CD40, a TNF receptor superfamily member, is expressed on various hematopoietic and non-hematopoietic cells [9, 10]. CD154 (CD40 ligand) is present on activated CD4^{+} T cells and platelets, and in plasma [9]. CD40–CD154 interaction not only regulates cellular and humoral immunity [9] but also activates inflammation. Indeed, CD40–CD154 signalling promotes atherosclerosis, graft rejection and various autoimmune disorders [11]. In vivo blockade of this pathway controls these diseases [11, 12]. Thus, the CD40–CD154 pathway is a target in the fight against various disorders in humans.

We explored the role of CD40 in the development of a microvascular complication of diabetes. We chose a model of retinopathy, since we reported that CD40 is constitutively

expressed on retinal endothelial cells, microglia and Müller cells, the main glia of the retina [13]. In addition, plasma CD154 levels are increased in patients with diabetic retinopathy [14].

Methods

Induction of diabetes in mice

Male C57BL/6 and *Cd40*$^{-/-}$ mice on B6 background (Jackson Laboratories, Bar Harbor, ME, USA) were rendered diabetic by administration of streptozotocin (STZ). Fasted mice of 20–25 g body weight received five daily i.p. injections of STZ (55 mg/kg; MP Biomedicals, Solon, OH, USA). Development of diabetes (blood glucose > 250 mg/ml) was assessed beginning 1 week after the first injection of STZ using a glucometer (Accu-Chek Aviva; Roche Diagnostics, Indianapolis, IN, USA). Glycated haemoglobin was measured every 2–3 months (VARIANT Classic; Bio-Rad, Hercules, CA, USA). Each group of diabetic and non-diabetic mice contained between eight and 12 animals. Mice were weighed weekly and received, if needed, 0–0.2 U NPH insulin s.c. up to three times per week. Insulin requirement was similar in diabetic B6 and *Cd40*$^{-/-}$ mice. All studies adhered to the institutional guidelines for humane treatment of animals, Principles of Laboratory Animal Care (National Institutes of Health [NIH], Bethesda, MD, USA), and to the Association for Research in Vision and Ophthalmology (ARVO) Statement for the Use of Animals in Ophthalmic and Vision Research.

Real-time quantitative PCR

cDNA was used as a template for real-time PCR using SYBR Green PCR Master Mix (Applied Biosystems, Foster City, CA, USA) and primers for ICAM-1 [15], CD40 [16] or 18S rRNA [15]. Expression of these genes was assessed using a 7300 Real-Time PCR System (Applied Biosystems). Each cDNA sample was run in duplicate. Samples were normalised according to the content of 18S rRNA.

Leucostasis

Fluorescein-coupled concanavalin A lectin (20% g/ml in PBS; Vector Laboratories, Burlingame, CA, USA) was infused as described [4, 17]. Retinal flat-mounts were viewed blindly via fluorescence microscopy, and brightly fluorescent leucocytes were counted in the entire retina.

Vascular histopathology

Retinal vasculature was isolated as described with minor modifications [17]. Eyes were fixed with 10% neutral buffered formalin followed by dissection of the retina. Retinas were incubated with elastase (40 U/ml; Calbiochem, San Diego, CA, USA). Neuroretinal tissue was brushed away and the vascular tree was stained with periodic acid-Schiff haematoxylin. Eight areas in the mid retina were examined blindly under ×400 magnification. Degenerate capillaries were defined as capillary-sized structures without any surrounding nuclei along their length. Approximately 1,000 capillary cells were examined blindly for the presence of pericyte ghosts. Pericyte ghosts were identified as spaces in the capillary basement membrane where pericytes had disappeared.

Immunohistochemistry

Zinc-fixed, paraffin-embedded eyes were treated with proteinase K for antigen retrieval. Four sections at different areas of the eyes were incubated with rat anti-CD40 monoclonal antibody (mAb) (3/23; BioLegend, San Diego, CA, USA), followed by incubation with tomato lectin DyLight 488 (Vector Laboratories), mouse anti-vimentin (Novus Biologicals, Littleton, CO, USA) or rabbit anti-ionised calcium-binding adapter molecule 1 (Iba-1) (Wako Chemicals, Richmond, VA, USA). Fluorescent secondary antibodies (Abs) were from Jackson ImmunoResearch Laboratories, West Grove, PA, USA. Specificity of staining was confirmed by omitting primary Abs. Retinas were analysed using a Leica DMI 6000 B automated microscope equipped for epifluorescence microscopy (Leica Microsystems, Buffalo Grove, IL, USA). Sections also were incubated with rabbit anti-nitrotyrosine Ab (Upstate Biotechnology, Lake Placid, NY, USA), followed by incubation with biotinylated secondary Ab (Jackson ImmunoResearch Laboratories). Sections were resolved using a Vectastain ABC kit (Vector Laboratories). Image analysis was performed in the innermost portion of the retina (the area of most intense immunoreactivity). Images were collected using an Olympus BX-60 upright microscope (Olympus America, Center Valley, PA, USA) equipped with a Retiga-EXL Aqua camera (QImaging, Vancouver, BC, Canada). A colour threshold representative of the staining was applied to the images. Same-size regions were drawn on anatomically matched areas of the retina (six fields per section). The area of tissue positively stained within these same-size regions was recorded. Samples were analysed blindly.

Cells

Human eyes from non-diabetic donors were obtained from the Cleveland Eye Bank. Primary human retinal endothelial cells [18] and Müller cells [19] were obtained as described. Endothelial cells were >90% positive for acetylated LDL. Human Müller cells were >95% positive for vimentin and cellular retinaldehyde-binding protein and negative for glial fibrillary acidic protein (GFAP). Human cells were treated with human CD154 (3 μg/ml; gift from W. Fanslow, Amgen, Thousand Oaks, CA, USA) or cell-free supernatant fractions containing multimeric CD154 (obtained from R. Kornbluth, Multimeric Biotherapeutics, La Jolla, CA, USA) for 24 h [15]. Both preparations gave similar results, and specificity was confirmed by detecting > 95% neutralisation by co-incubation with anti-human CD154 mAb. Omission of CD154 or a non-functional CD154 mutant [20] (T147N) was used as control. A mouse cell line with Müller cell characteristics [21] was incubated for 24 h with a stimulatory anti-CD40 mAb (1C10; 10 μg/ml), control mAb (BD Biosciences, San Jose, CA, USA) or either cell-free supernatant fraction containing mouse CD154 or inactive CD154. Studies with human cells were approved by the Institutional Review Board.

Flow cytometry

Retinas from each mouse were pooled and cell suspensions were obtained for flow cytometry as described [13]. Retinas were digested with papain (15 IU/ml)/DNAse (15 μg/ml; Worthington Biochemicals, Freehold, NJ, USA). Tissue was passed through a 40 μm cell strainer. Tissue trapped by the strainer was digested with collagenase type I (1 mg/ml; Worthington Biochemicals) to free endothelial cells. Cells obtained after papain/DNase and

collagenase treatment were pooled. Cell suspensions that were >90% unicellular and with cell viability >90% were incubated with anti-CD11b (eBioscience, San Diego, CA, USA), anti-CD31 (eBioscience), anti-thymus cell antigen 1 (Thy-1) (to detect ganglion neurons; eBioscience) or isotype control Abs. For detection of intracellular markers, cells were treated with IntraPrep permeabilisation reagent (Beckman Coulter, Hialeah, FL, USA) and stained with anti-vimentin (to detect Müller cells; Santa Cruz Biotechnologies, Santa Cruz, CA, USA), anti-GFAP (this marker identifies astrocytes; Santa Cruz Biotechnologies) or isotype control Abs. Cells were co-stained with anti-CD40 mAb (BD Biosciences). Flow cytometry data were acquired from 5×10^5 to 1×10^6 cells using an LSR II and running FACSDiva software (BD Biosciences). WinList software (Verity Software House, Topsham, ME, USA) was used for data analysis. Expression of CD40 was analysed on gated $CD11b^+$, $CD31^+$, $vimentin^+$, $Thy\text{-}1^+$ or $GFAP^+$ cells. CD40 expression is expressed as corrected mean fluorescence intensity (cMFI) obtained by subtracting fluorescence observed with isotype control mAb from that obtained with anti-CD40 mAb. Human cells were incubated with anti-ICAM-1 (eBioscience) or isotype control mAbs.

Immunoblot

Membranes were probed with Ab to ICAM-1 (Santa Cruz Biotechnologies), NOS2 (BD Biosciences) or actin (Santa Cruz Biotechnologies), followed by incubation with secondary Ab conjugated to horseradish peroxidase (Santa Cruz Biotechnologies). Intensities of ICAM-1 were calculated using ImageJ (NIH) and normalised against actin.

Measurement of nitrite production

Nitrite concentrations were calculated using Griess reaction (Promega, Madison, WI, USA).

Statistical analysis

All results are expressed as the mean ± SEM. Statistical significance of in vitro experiments was analysed by two-tailed Student's *t* test or ANOVA. In vivo experiments were analysed using the non-parametric Kruskal–Wallis test followed by the Mann–Whitney *U* test. Differences were considered statistically significant at $p<0.05$.

Results

Diabetic $Cd40^{-/-}$ mice have defective upregulation of pro-inflammatory molecules in the retina and impaired leucostasis

Male B6 and $Cd40^{-/-}$ mice were rendered diabetic by administration of STZ. Blood glucose concentrations, HbA_{1c} levels and body weights of diabetic B6 and $Cd40^{-/-}$ mice were similar (Table 1) ($p>0.5$). While upregulation of inflammatory responses can be detected in the retina within the first 2 months of diabetes in mice, retinal capillary degeneration takes longer to develop and is typically examined at 8 months of diabetes in mice [2, 7]. Unless otherwise stated, animals were examined at 2 months of diabetes to assess expression of pro-inflammatory molecules and leucostasis, or at 8 months of diabetes to examine vascular histopathology. Compatible with a low-grade chronic inflammation, leucocyte adhesion to the retinal vasculature (leucostasis) is increased in retinal vessels in diabetic animals [4, 22]. The retinal microvasculature of diabetic B6 mice exhibited the expected significant increase

in the number of adherent leucocytes compared with non-diabetic mice (Fig. 1). Diabetic *Cd40*$^{-/-}$ mice had a significant reduction in the number of adherent leucocytes compared with diabetic B6 animals (Fig. 1). Next, we determined whether CD40 is required for ICAM-1 upregulation in the retinas of diabetic mice. We examined *Icam1* mRNA levels, since ICAM-1 expression is controlled at the mRNA level [23]. mRNA levels of *Icam1* were similar in non-diabetic B6 and *Cd40*$^{-/-}$ mice (Fig. 2). Diabetic B6 mice upregulated mRNA levels of *Icam1* in the retina (Fig. 2), as reported in diabetic rodents [24]. By contrast, *Icam1* upregulation was significantly impaired in diabetic *Cd40*$^{-/-}$ mice (Fig. 2a). Similar results were observed when ICAM-1 protein expression was examined in retinal lysates (Fig. 2b). We also examined ICAM-1 protein expression at 6 months of diabetes. Whereas diabetic B6 mice exhibited a 2.5 ± 0.4-fold increase in ICAM-1 levels compared with non-diabetic B6 mice, ICAM-1 expression was 1.1 ± 0.2-fold higher in diabetic *Cd40*$^{-/-}$ mice compared with non-diabetic *Cd40*$^{-/-}$ mice ($p<0.01$). Thus, diabetic *Cd40*$^{-/-}$ mice exhibit diminished ICAM-1 upregulation and leucostasis in the retina; responses linked to the development of diabetic retinopathy.

CD40 is upregulated in the retina of diabetic mice

CD40 upregulation is a common feature of diseases driven by CD40 [25]. To further explore the role of CD40 in diabetic retinopathy we examined CD40 expression in the retina. B6 mice that had been diabetic for 2 months exhibited increased *Cd40* mRNA levels compared with non-diabetic controls (Fig. 3). Next, we examined CD40 protein expression at a cellular level. Flow cytometry effectively quantifies upregulation of molecules in primary retinal cells in retinal inflammation [13]. We obtained retinal cell suspensions from non-diabetic and diabetic B6 mice and examined CD40 expression by flow cytometry. CD40 is expressed in various non-leucocytes at levels that are typically low but nevertheless functional [9, 26]. Confirming our previous report [13], retinal endothelial cells, Müller cells, microglia and ganglion neurons expressed CD40 under basal conditions (Fig. 4). There was a moderate but significant upregulation of CD40 in endothelial cells (CD31^{+}), Müller cells (vimentin^{+}) and microglia/macrophage (CD11b^{+}) at 2 months of diabetes. Of note, even a modest CD40 upregulation remarkably potentiates responses triggered by CD40 ligation [27]. Ganglion neurons (Thy-1^{+}) did not exhibit a significant increase in CD40 expression (Fig. 4), and astrocytes (GFAP^{+}) remained CD40^{-} (data not shown). Studies performed at 6 months of diabetes also revealed that, compared with controls, diabetic B6 mice exhibited increased CD40 levels in CD31^{+}, vimentin^{+} and CD11b^{+} retinal cells (1.8 ± 0.2, 2.1 ± 0.3 and 2.4 ± 0.1, respectively; $p<0.05$). Immunohistochemistry studies were conducted to further examine CD40 expression. Retinas from diabetic and non-diabetic B6 mice were stained with Ab against CD40, vimentin or Iba-1 (microglia/macrophages), or with tomato lectin (endothelial cells). Compared with non-diabetic B6 mice, tomato lectin^{+}, vimentin^{+} and Iba-1^{+} cells from diabetic animals exhibited increased expression of CD40 (Fig. 5). Thus, diabetic mice exhibit upregulation of CD40 in the retina.

Primary retinal endothelial cells and Müller cells upregulate ICAM-1 in response to CD40 ligation

We examined whether CD40 engagement modulates ICAM-1 expression in primary retinal cells. Primary human retinal endothelial cells and Müller cells were incubated with CD154,

a non-functional CD154 mutant (T147N) [21] or culture medium alone. ICAM-1 expression was similar on cells incubated with medium alone or with non-functional CD154 (not shown). By contrast, stimulation with CD154 significantly upregulated ICAM-1 in retinal endothelial cells and Müller cells (Fig. 6).

CD40 promotes nitration of retinal proteins and upregulates NOS2 and nitric oxide production by Müller cells

Increased production of peroxynitrate results in nitration of tyrosine; thus, detection of nitrotyrosine is a marker of nitrosative stress in the diabetic retina. Nitrotyrosine expression is increased in the retinas of humans and rodents with diabetes [6, 8, 28–30]. This increase has been proposed to occur at the level of the retinal vasculature and is detected more intensely in the inner retina [6, 8, 28–30]. Moreover, increased nitrotyrosine expression persists for several months in the retina of diabetic rodents [31]. Retinas from B6 mice that had been diabetic for 2 or 8 months revealed increased staining for nitrotyrosine mainly in the innermost portion of the retina (Fig. 7a; and data not shown). By contrast, diabetic *Cd40*$^{-/-}$ mice showed diminished staining for nitrotyrosine (Fig. 7a, b). Müller cells from patients with diabetic retinopathy appear to express NOS2 [5]. We determined whether CD40 ligation affects NOS2 expression and production of nitric oxide by Müller cells. Primary human Müller cells upregulated NOS2 in response to stimulation with CD154 (Fig. 8a). Incubation with a stimulatory anti-CD40 mAb upregulated NOS2 in a mouse Müller cell line (Fig. 8b) and caused increased nitrite production (Fig. 8c). Similar results were obtained when mouse Müller cells were incubated with mouse CD154 (data not shown). Taken together, CD40 drives retinal protein nitration in vivo and causes Müller cells to upregulate NOS2 and produce nitric oxide, which are responses linked to the development of diabetic retinopathy.

Diabetic Cd40$^{-/-}$ mice are protected against retinal vascular degeneration

Loss of endothelial cells and pericytes with resulting formation of degenerate capillaries is a key feature of diabetic retinopathy. We examined whether CD40 promotes the development of capillary degeneration. At 8 months of diabetes, B6 diabetic mice exhibited the expected significant increase in the number of degenerate capillaries and pericyte `ghosts' (a marker of loss of pericytes) compared with non-diabetic animals (Fig. 9). By contrast, diabetic *Cd40*$^{-/-}$ mice did not develop capillary degeneration or pericyte loss (Fig. 9). Thus, CD40 is important for the development of signature vascular features of diabetic retinopathy.

Discussion

We report that diabetic *Cd40*$^{-/-}$ mice exhibited a decrease in numerous key responses linked to the pathogenesis of diabetic retinopathy including leucostasis, ICAM-1 upregulation and nitration of retinal proteins; furthermore, these animals were protected from development of retinal capillary degeneration. CD40 upregulation occurred in the retinas of diabetic mice. Moreover, in vitro studies further supported a pathogenic role of CD40 in early diabetic retinopathy, since CD40 ligation upregulated ICAM-1 on retinal endothelial cells and Müller cells and upregulated NOS2/nitric oxide production by Müller cells. Our work

suggests that CD40 is an upstream regulator of various responses involved in the development of a microvascular complication of diabetes.

Our demonstration that CD40 promotes in vivo and in vitro upregulation of ICAM-1 and leucostasis may explain why *Cd40*$^{-/-}$ mice are protected from capillary degeneration. However, it has been suggested that leucostasis may not solely explain capillary degeneration. While diabetic mice deficient in 5-lipoxygenase exhibited diminished leucostasis and were protected from capillary degeneration, animals deficient in 12-lipoxygenase showed no reduction in capillary degeneration despite ablation of leucostasis [22]. Thus, there may be additional mechanisms besides leucostasis by which CD40 promotes vascular injury. Other mediators that promote retinal capillary degeneration in diabetes include NOS2 and oxidative stress [7, 32]. We report that CD40 stimulates NOS2 upregulation as well as nitric oxide production by retinal Müller cells, and CD40 is reported to upregulate nitric oxide production by microglia [33]. Moreover, CD40 ligation can stimulate production of reactive oxygen species [34]. Whether nitrosative and/or oxidative stress contributes to the role of CD40 in ICAM-1 upregulation remains to be determined.

Müller cells in diabetes exhibit disrupted homeostatic functions, upregulate pro-inflammatory cytokines and acquire a reactive phenotype (GFAP upregulation) [35, 36]. In addition, NOS2 appears to be expressed in Müller cells from patients and animals with diabetic retinopathy [5, 6]. The mechanisms that explain Müller cell dysfunction in diabetes remain poorly understood. The breakdown of the blood–retinal barrier detected in diabetes raises the possibility that CD154 could interact with CD40^{+} Müller cells. We report that CD40-stimulated Müller cells upregulate NOS2, produce nitric oxide and upregulate ICAM-1. Of potential relevance to CD40-induced ICAM-1 upregulation on Müller cells is the report that retinas from patients with diabetic retinopathy exhibit ICAM-1 upregulation throughout the retina [37].

Peroxynitrate production is associated with accelerated neuronal death in diabetic retinopathy [28, 30]. Our studies indicate that CD40 drives protein nitration in the diabetic retina, raising the possibility that CD40 may promote retinal neurodegeneration in diabetes. Indeed, in a mouse model of retinopathy induced by ischaemia/reperfusion, *Cd40*$^{-/-}$ mice exhibited diminished loss of ganglion cells [15]. Our in vitro studies also suggest that Müller cells are involved in the protein nitration driven by CD40 in the diabetic retina. However, it is likely that CD40 promotes nitrosative stress by also acting on other cells. CD40 is expressed on retinal microglia [13], which appear to become activated in diabetic retinopathy [38]. Moreover, CD40 upregulates NOS2 and nitric oxide production by microglia [33].

The CD40–CD154 pathway is likely activated in diabetes. Our studies revealed that CD40 is upregulated on retinal endothelial cells, Müller cells and microglia of diabetic mice. There is evidence that CD154 is increased in diabetes. Membrane CD154 on activated platelets triggers pro-inflammatory responses in endothelial cells [39]. The evidence for microthrombosis in the retinal capillaries of patients and animals with diabetic retinopathy [40] suggests the existence of increased local levels of CD154. CD154 also exists as a biologically active soluble homotrimer released primarily by activated platelets [41]. The

levels of soluble CD154 in the circulation are increased in diabetic individuals, especially those with microangiopathy [14, 42, 43]. Moreover, serum CD154 from diabetic individuals induces pro-inflammatory responses in endothelial cells and monocytes [43]. Thus, there is not only increased CD40 expression in the retina in diabetes but also a likely local and systemic source of CD154 that can drive CD40-dependent responses in the retina during diabetes.

Of likely relevance to diabetic retinopathy, there is crosstalk between CD40 and TNF-α/IL-1β. CD40 cooperates with IL-1β for chemokine production by endothelial cells [15], and CD40 and TNF-α synergise to upregulate NOS2 in macrophages [44]. CD40 enhances TNF-α and IL-1β production [45], and TNF-α and IL-1 can increase CD40 expression [26]. While CD40 may act in concert with TNF-α and IL-1 in the development of diabetic retinopathy, our studies indicate that deletion of CD40 is sufficient to blunt the development of this disease.

In summary, these studies indicate an important role of CD40 in the development of microvascular complications of diabetes. Given that targeting a single molecule (CD40) would impair the activation of many mediators of this disease, this work suggests that the levels of glycaemic control achieved with current treatment for diabetes plus approaches to inhibit CD40 signalling may result in improved management of diabetic retinopathy.

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Abbreviations

Ab	Antibody
CD	Cluster of differentiation
cMFI	Corrected mean fluorescence intensity
GFAP	Glial fibrillary acidic protein
Iba-1	Ionised calcium-binding adapter molecule 1
ICAM-1	Intercellular adhesion molecule 1
mAb	Monoclonal antibody
NIH	National Institutes of Health
NOS2	Nitric oxide synthase 2

STZ	Streptozotocin
Thy-1	Thymus cell antigen 1

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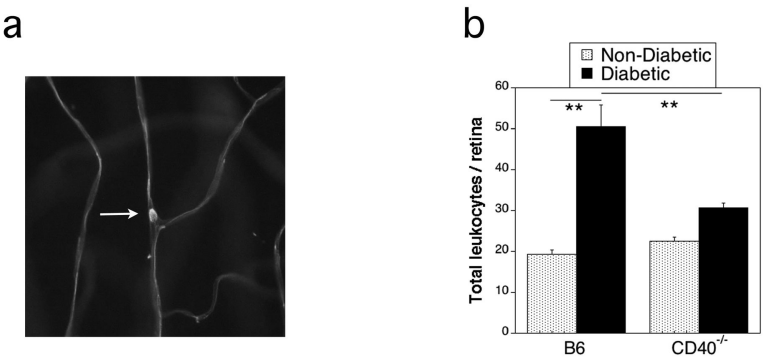

Fig. 1.

CD40 promotes leucostasis in the retinas of diabetic mice. Adherent leucocytes in the retinal vasculature of diabetic and non-diabetic control mice were quantified. (**a**) Representative image of an adherent leucocyte within the vasculature of a diabetic B6 mouse. Bar, 50 μm. (**b**) Quantification of adherent leucocytes per retina (ten to 12 mice per group). White bars, non-diabetic mice; black bars, diabetic mice. ***p*<0.01

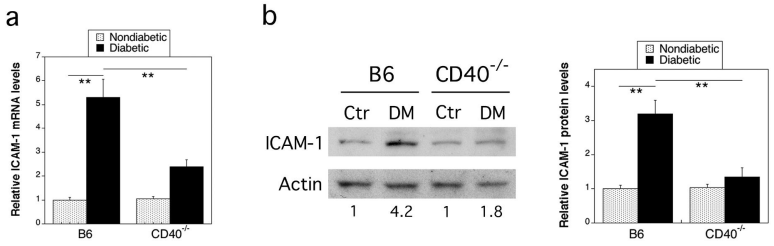

Fig. 2.

Diabetic *Cd40*$^{-/-}$ mice have impaired ICAM-1 upregulation. (**a**) mRNA levels of *Icam1* were assessed by real-time quantitative PCR. (**b**) ICAM-1 and actin expression by immunoblot. Each lane corresponds to a representative mouse. Numbers under immunoblot represent relative expression of ICAM-1 after normalisation to actin for the representative mouse. Bar graph represents average ± SEM of relative expression of ICAM-1 (fold vs control for the same genotype) after normalisation to actin. Non-diabetic mice were given an arbitrary number of 1 (12–15 mice per group). White bars, non-diabetic mice (Ctr); black bars, diabetic mice (DM). ***p*<0.01

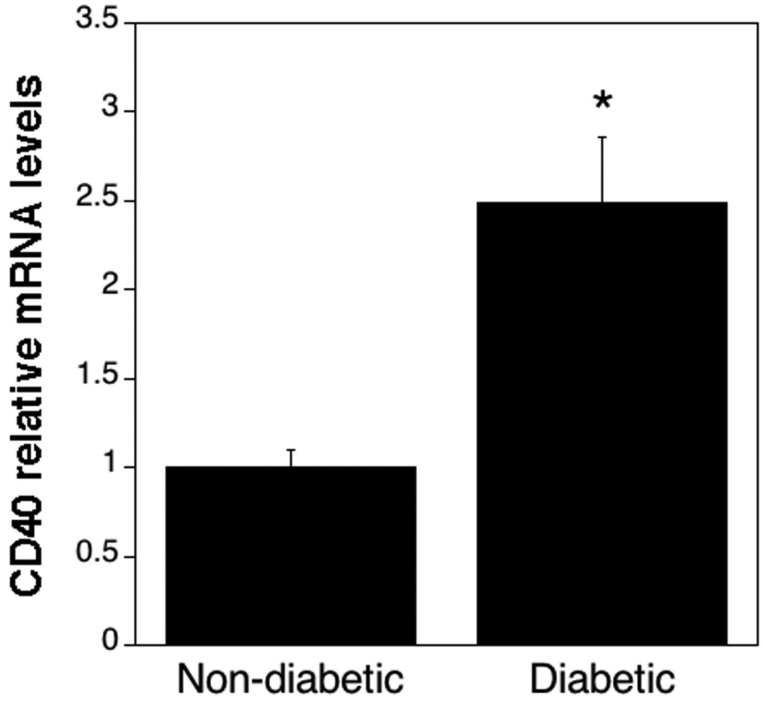

Fig. 3.

CD40 is upregulated in the retina of diabetic mice. *Cd40* mRNA levels were assessed by real-time quantitative PCR. Data are expressed as fold increase in diabetic mice compared with retinas from non-diabetic controls (15–18 mice per group). *$p<0.05$

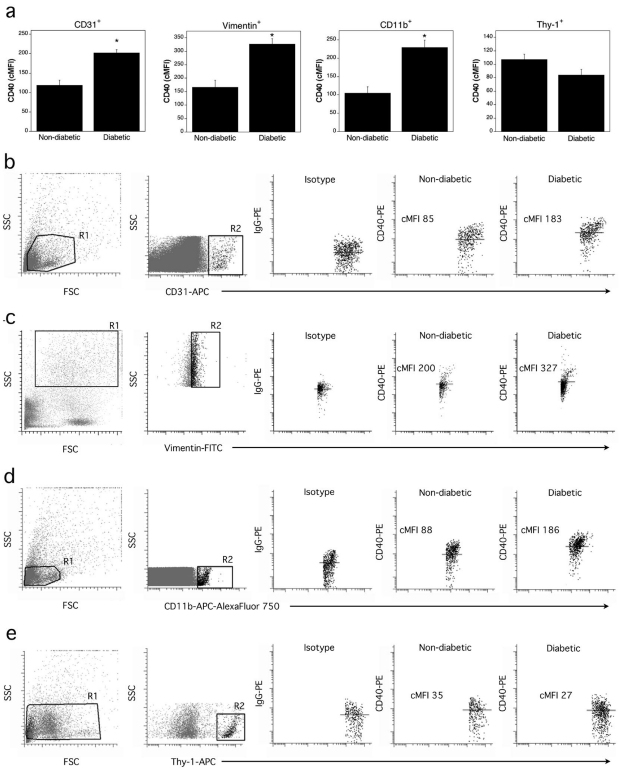

Fig. 4.

CD40 is upregulated in the retina of diabetic mice. Retinal cells were incubated with anti-CD40 mAb plus either anti-CD31 mAb, anti-vimentin Ab, anti-CD11b mAb or anti-Thy-1 mAb. (**a**) cMFI for CD40 on gated $CD31^+$, $vimentin^+$, $CD11b^+$ or $Thy\text{-}1^+$ cells. cMFI was calculated by subtracting fluorescence obtained with isotype control mAb from that obtained with anti-CD40 mAb. Data shown are representative of one of four independent experiments. (**b–e**) Strategy for analysis of CD40 expression. Dot plots of forward scatter (FSC) vs side scatter (SSC) show gates for endothelial cells (**b**), Müller cells (**c**), macrophage/microglia (**d**) or ganglion neurons (**e**) (R1), drawn based on published patterns of FSC and SSC for these cells [13]. After gating on R1, dot plots of SSC vs CD31, vimentin, CD11b or Thy-1 were obtained and gates encompassing $CD31^+$, $vimentin^+$, $CD11b^+$ or $Thy\text{-}1^+$ cells were generated (R2). Dot plots show either isotype control or CD40 expression on $CD31^+$, $CD11b^+$, $vimentin^+$ or $Thy\text{-}1^+$ cells. Fluorescence is shown in logarithmic scale (base 10) (ten mice per group). APC, allophycocyanin; FITC, fluorescein isothiocyanate; PE, phycoerythrin. *$p<0.05$

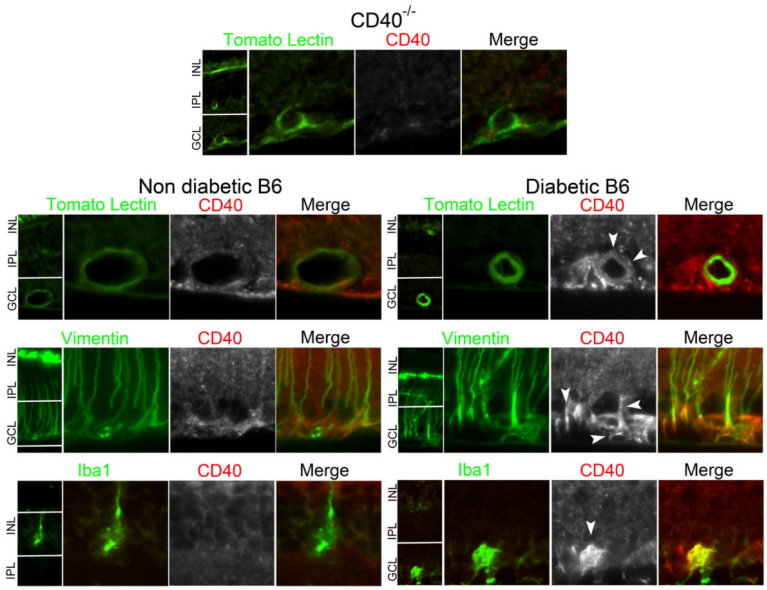

Fig. 5.

CD40 is upregulated in the retina of diabetic mice. Sections from B6 mice at 8 months of diabetes and non-diabetic B6 mice were incubated with anti-CD40 mAb plus tomato lectin, anti-vimentin mAb or anti-Iba-1 Ab. A section from a *Cd40*$^{-/-}$ mouse is shown as a negative control. White boxes represent areas that were magnified. GCL, ganglion cell layer; INL, inner nuclear layer; IPL, inner plexiform layer (original magnification ×400; six to seven mice per group)

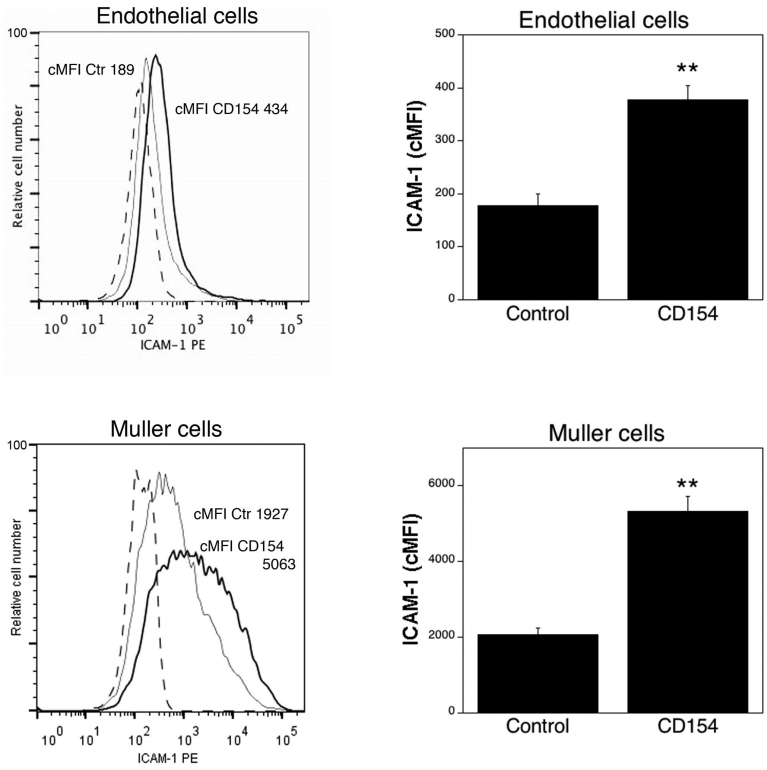

Fig. 6.

Primary human retinal endothelial cells and Müller cells upregulate ICAM-1 in response to CD40 ligation. Endothelial cells (**a, b**) and Müller cells (**c, d**) were incubated with human CD154 or a non-functional CD154 mutant (Ctr). Histograms: dashed line, isotype control; grey line, control; black line, CD154. Bar graph represents cMFI (mean ± SEM). Data are representative of three experiments. ***p*<0.01

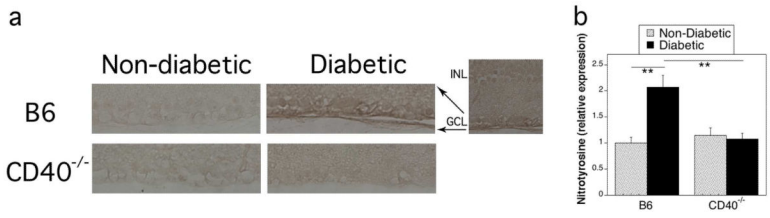

Fig. 7.

Diabetic *Cd40*$^{-/-}$ mice have diminished expression of nitrotyrosine in the retina. Nitrotyrosine was detected more intensely in the innermost area of the retina of diabetic B6 mice (**a**). Original magnification ×400. No staining was detected when using secondary Ab alone. (**b**) Semiquantification for nitrotyrosine immunohistochemistry staining. Image analysis was performed in the innermost portion of the retina. Same-size regions were drawn on anatomically matched areas of the retina (six fields per section). Signal intensity was determined as described in the Methods section and given an arbitrary score of 1 for non-diabetic B6 mice (ten mice per group). GCL, ganglion cell layer; INL, inner nuclear layer. **$p<0.01$

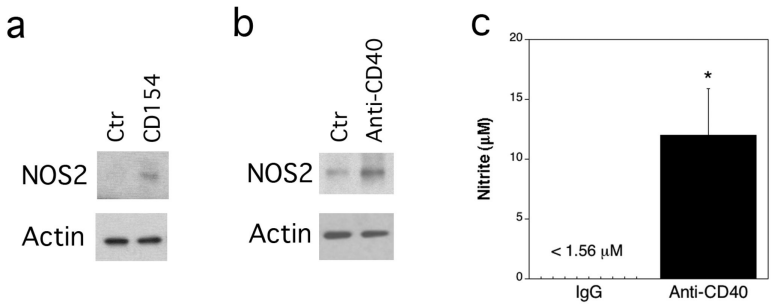

Fig. 8.

Müller cells upregulate NOS2 and produce nitric oxide in response to CD40 ligation. (**a**) Primary human Müller cells were incubated with human CD154 or a nonfunctional CD154 mutant (Ctr). (**b**, **c**) Mouse Müller cells were incubated with a stimulatory anti-CD40 or control mAb. NOS2 expression was examined by immunoblot (**b**). Nitric oxide concentrations in supernatant fractions (**c**). Data are representative of three to four experiments. *p<0.05

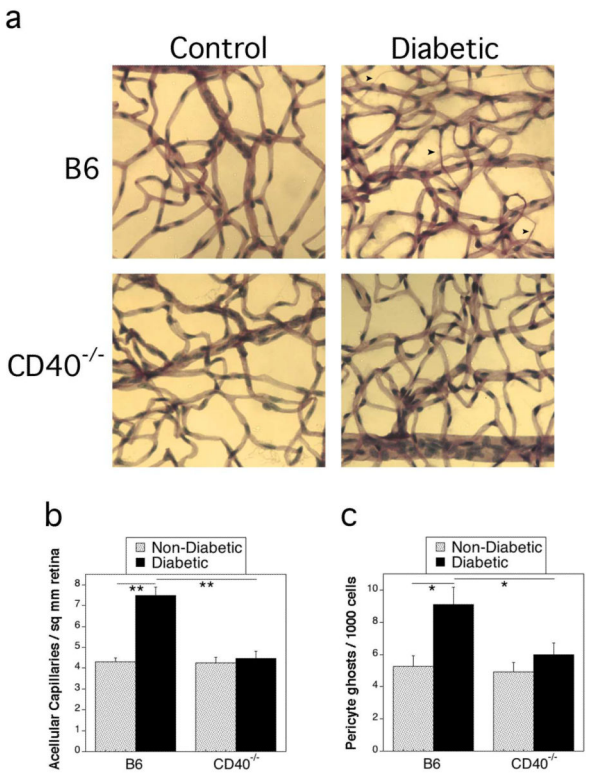

Fig. 9.

Diabetic *Cd40*$^{-/-}$ mice are protected against retinal vascular degeneration. Retinal digests were examined for the presence of degenerate capillaries (**a**, **b**). Arrows show representative degenerate capillaries (**a**). Original magnification ×400. Digests were examined for the presence of pericyte ghosts (**c**). Data shown represent mean ± SEM (12–14 mice per group). **p*<0.05, ***p*<0.01

Table 1

Average weight, blood glucose and HbA_{1c} levels in control and diabetic mice

Strain	Group	Duration (months)	*n*	Weight (g)	Glucose (mg/ml)	HbA_{1c} (%)	HbA_{1c} (mmol/mol)
B6	Control	2	55	34.0±0.45	149.5±3.31	3.0±0.04	
		6	17	37.4±0.81	114.8±13.48	3.1±0.07	
		8	37	42.1±0.86	155.2±4.91	3.5±0.05	
	Diabetic	2	51	29.0±0.34	412.5±12.68	8.9±0.15	73.8
		6	16	25.9±1.10	356.5±50.1	9.9 ± 0.44	84.7
		8	32	27.72±0.63	446.6±21.03	10.0±0.35	85.8
Cd40 $^{-/-}$	Control	2	25	33.0±0.75	159.0±6.52	3.1±0.06	
		6	18	35.1±0.81	121.2±12.88	3.0±0.06	
		8	34	42.33±0.93	149.5±4.60	3.5±0.04	
	Diabetic	2	33	26.0±0.41	463.0±11.70	9.0±0.17	74.9
		6	18	26.3 ± 0.70	356.0±51.59	9.9±0.38	84.7
		8	20	25.6±0.48	373.2±13.48	8.7±0.51	71.6