Interleukin-6 Production in CD40-Engaged Fibrocytes in Thyroid-Associated Ophthalmopathy: Involvement of Akt and NF- κ B

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PURPOSE. CD40-CD40 ligand (CD40L) interactions appear to play pathogenic roles in autoimmune disease. Here we quantify CD40 expression on fibrocytes, circulating, and bone marrow-derived progenitor cells. The functional consequences of CD40 ligation are determined since these may promote tissue remodeling linked with thyroid-associated ophthalmopathy (TAO).

METHODS. CD40 levels on cultivated fibrocytes and orbital fibroblasts (GOFB) from patients with Graves' disease (GD), as well as fibrocyte abundance, were determined by flow cytometry. CD40 mRNA expression was evaluated by real-time PCR, whereas response to CD40 ligation was measured by Luminex and RT-PCR. Protein kinase B (Akt) and nuclear factor (NF)- κ B (NF- κ B) signaling were determined by Western blot and immunofluorescence.

RESULTS. Basal CD40 expression on fibrocytes is greater than that on GOFB. IFN- γ upregulates CD40 in both cell types and its actions are mediated at the pretranslational level. Fibrocytes produce high levels of cytokines, including interleukin-6 (IL-6), TNF- α , IL-8, MCP-1, and RANTES (Regulated on Activation, Normal T Cell Expressed and Secreted) in response to CD40L. IL-6 induction results from an increase in steady state IL-6 mRNA, and is mediated through Akt and NF- κ B activation. Circulating CD40⁺CD45⁺Col1⁺ fibrocytes are far more frequent in vivo in donors with TAO compared with healthy subjects.

CONCLUSIONS. Particularly high levels of functional CD40 are displayed by fibrocytes. CD40L-provoked signaling results in the production of several cytokines. Among these, IL-6 expression is mediated through Akt and NF- κ B pathways. The frequency of circulating CD40⁺ fibrocytes is markedly increased in patients with TAO, suggesting that this receptor might represent a therapeutic target for TAO. (*Invest Ophthalmol Vis Sci.* 2012;53:7746-7753) DOI:10.1167/iov.12-9861

CD40, a member of the tumor necrosis factor- α (TNF- α) receptor superfamily, is a cell surface determinant originally identified on B cells.¹ It has been detected on several cell types, including monocytes, macrophages, dendritic cells, and fibroblasts.²⁻⁵ CD40 activation, which occurs through its engagement with CD40 ligand (CD40L, also known as CD154), appears to participate in the pathogenesis of several autoimmune diseases.⁶⁻⁹ CD40L is transiently expressed on the surface of activated T cells, but can also be detected on other cell types, including fibroblasts.¹⁰ Aberrant CD40-CD40L interactions in medullary thymic epithelial cells might contribute to autoimmunity by disrupting central immune tolerance.¹¹

Graves' disease (GD) is the most common form of hyperthyroidism in North America. Thyroid growth, remodeling, and overactivity result from stimulatory autoantibodies generated against the thyrotropin receptor (TSHR) coupled with lymphocytic infiltration. GD can also be manifest by ocular changes or thyroid-associated ophthalmopathy (TAO), a process in which the orbit becomes inflamed and undergoes remodeling.¹² The pathogenesis of TAO is incompletely understood, a void that has hindered development of effective therapeutic agents.¹³ Central to the immune reactivity in the orbit are the unique phenotypic attributes displayed by fibroblasts.^{14,15} These heterogeneous cells respond to proinflammatory cytokines, including IL-1 β , leukoregulin, TGF- β , and CD40L.^{16,17} Orbital fibroblasts display high levels of cell-surface CD40 when exposed to interferon gamma (IFN- γ).¹⁶ CD40 ligation in these cells results in production of IL-8, IL-6, and monocyte chemoattractant protein-1 (MCP-1), transcriptional activation of the prostaglandin endoperoxide H synthase 2 (*PGHS-2*) gene, and the production of high levels of prostaglandin E₂ and hyaluronan.^{16,18,19} The CD40/CD40L bridge provides a putative pathway through which fibroblast/T-cell cross-talk can promote tissue reactivity.¹⁶

Fibrocytes are bone marrow-derived progenitor cells that can migrate to sites of tissue inflammation and express surface markers characteristic of both fibroblasts (collagen type I [Col1]) and hematopoietic cells (CD34 and CD45).^{20,21} Fibrocytes are capable of presenting antigen and secreting several cytokines. They participate in inflammatory and profibrotic immune processes.^{9,22,23} Douglas et al.²⁴ reported

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an increased abundance of fibrocytes cultivated from peripheral blood mononuclear cells (PBMCs) of patients with GD compared with healthy controls. Furthermore, CD34⁺ fibroblasts can be identified easily in orbital tissues and among orbital fibroblasts from patients with TAO. They are absent in healthy orbital tissues and fibroblast strains derived from normal tissues.²⁴ Recently, we demonstrated high levels of TSHR displayed by fibrocytes, regardless of whether they emanate from healthy donors or those with GD. These cells respond to TSH and monoclonal activating antibodies directed against TSHR to produce proinflammatory cytokines.²⁵ In addition, fibrocytes infiltrate the thyroid in GD.¹⁵ We postulate that circulating CD40⁺ fibrocytes infiltrate the orbit and thyroid in TAO and GD and participate in disease pathogenesis.

Here, we provide the first evidence that functional CD40 is displayed on circulating fibrocytes. The population of fibrocytes is skewed in TAO toward a CD40⁺ phenotype. Further, protein kinase B (Akt) and nuclear factor- κ B (NF- κ B) signaling appear to mediate the upregulation of interleukin-6 (IL-6) following CD40 engagement. These findings suggest a mechanism through which fibrocytes might cross-talk with CD40L-bearing T cells. CD40 display and function on fibrocytes may serve as clinically useful biomarkers and could be therapeutically targeted in TAO.

METHODS

Patient Samples

Patients with TAO ($n = 23$) and healthy donors ($n = 19$) were recruited consecutively from the patient population of Kellogg Eye Center at the University of Michigan. Exclusion criteria included: current or recent (within the last 6 months) immunosuppression, other autoimmune diseases, asthma, chronic inflammation, human immunodeficiency virus, recent trauma, or active infection. Informed consent was obtained in compliance with policies of the Institutional Review Board of the University of Michigan Health System. Research methods followed the tenets of the Declaration of Helsinki. Median age was 51 years for TAO and 42 years for healthy subjects. Most subjects were female (TAO, $n = 16$, 70%; healthy control, $n = 10$, 71%) and Caucasian (TAO, $n = 18$, 78%; healthy control, $n = 12$, 86%). Median duration of TAO was 2.0 years, ranging from 0.5 to 36.0 years. The majority of patients were in the inactive (stable) phase (Clinical Activity Score [CAS] ≤ 3 , $n = 18$, 78%) and had extraocular muscle dysfunction ($n = 14$, 61%). Among subjects with TAO, 22 had a history of hyperthyroidism, with a median duration of 5.5 years, ranging from 0.5 to 39.0 years. All participants were euthyroid at the time of study participation. Graves' orbital fibroblast (GOFB) strains were initiated from intraconal orbital fat obtained from waste during surgical decompression as described previously.²⁶

Cell Culture and Treatments

Fibrocytes were cultivated by subjecting PBMCs to culture conditions similar to those described by Bucala et al.²⁰ Briefly, they were isolated from blood by centrifugation over density gradient cell separation medium (Histopaque-1077; Sigma-Aldrich, St. Louis, MO). Each well of a 24-well plate was inoculated with 5×10^6 cells in Dulbecco's modified Eagle's medium (DMEM) with 10% fetal bovine serum (FBS). After 7 days in culture, nonadherent cells were removed by gentle aspiration. Medium was replaced every 3 to 4 days. After 12 to 14 days in culture, adherent cells (<5% of the initial PBMC population) were washed and removed from the substratum by scraping. Culture purity was verified to be >90% fibrocytes by flow cytometry (fluorescence-activated cell sorting [FACS]) analysis and cell viability was >90% by trypan blue exclusion.

GOFB strains were initiated and cultivated as described previously.²⁶ All experiments were performed between the second and twelfth passages from culture initiation. We have previously determined that these GOFB strains are free from contamination with epithelial, endothelial, or smooth muscle cells and maintain a stable phenotype over this culture interval.^{27,28}

Cultivated fibrocytes and GOFB were treated with the test agents indicated in the figure legends. A day prior to treatment, the culture medium was changed to DMEM with 1% FBS. IFN- γ (Peprotech, Rocky Hill, NJ) was added to cultures (500 IU/mL final concentration). CD40L recombinant membrane or control membrane was kindly provided by Richard Phipps (University of Rochester) or purchased as a high activity construct (MegaCD40L; Enzo Life Sciences, Farmingdale, NY) and added to the culture medium at a final concentration of 100 ng/mL.

In some experiments, cells were pretreated with 50 nM AKT inhibitor IV (Calbiochem, Gibbstown, NJ), carbobenzoxy-Leu-Leu-leucinal (MG132) (10 nM) (Cayman, Ann Arbor, MI), or pyrrolidine dithiocarbamate (PDTc) (300 nM; Sigma-Aldrich) for 1 hour.

mRNA Quantification by RT-PCR

CD40 and IL-6 mRNA levels were monitored by real-time PCR in fibrocytes at several time points following the treatment intervals indicated. RNA was processed using a commercial purification kit (RNeasy Mini Kit; Qiagen, Valencia, CA) and reverse transcribed using a reverse transcription kit (Quantitect Reverse Transcription Kit; Qiagen). Quantitative PCR was performed on a thermocycler (CFX96; Bio-Rad Laboratories, Hercules, CA) using the Quantifast SYBR Green kit (Qiagen) using the following primers for CD40: 5'-AGA GTT CAC TGA AAC GGA ATG CC-3' (forward primer); 5'-ACA GGA TCC CGA AGA TGA TGG-3' (reverse primer). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as the housekeeping gene control, using 5'-TTG CCA TCA ATG ACC CCT-3' (forward primer) and 5'-CGC CCC ACT TGA TTT TGG-3' (reverse primer). IL-6 primers were purchased from Qiagen.

Cytokine Assay

Culture media of untreated and treated cells were collected and subjected to cytokine analysis (Cytokine Human 30-Plex Panel and IL-6 Human Singleplex Bead Kits, cat #LHC6003 and #LHC0061; Life Technologies, Grand Island, NY).

Immunofluorescence Microscopy

Fibrocytes were cultivated on 8-chamber slides for 12 to 14 days. For CD40 immunofluorescence, cells were harvested and fixed in 4% paraformaldehyde, blocked with goat serum (5%) for 30 minutes at room temperature, and incubated with primary mouse anti-human CD40 (cat #611362; BD Biosciences, San Jose, CA) or fluorescein isothiocyanate (FITC) goat anti-mouse Ig isotype control, cat #554001; BD Biosciences) overnight at 4°C. After three washes in phosphate-buffered saline (PBS) containing 0.1% Tween 20, slides were incubated with secondary antibodies for 1 hour at room temperature in the dark. For detecting NF- κ B p65 immunofluorescence, fibrocytes were incubated with CD40L (100 ng/mL) or control membrane for 30 minutes at 37°C. The cells were washed twice with PBS and fixed. Cells were air-dried and permeabilized with 100% methanol. Nonspecific antibody binding was blocked with 3% bovine serum albumin (BSA) in PBS. Cells were incubated overnight at 4°C with antibodies to the subunit (cat #ab16502; 1:400 dilution in PBS containing 3% BSA; Abcam, Cambridge, MA), washed, and then incubated at room temperature first for 30 minutes with secondary antibodies (Alexa Fluor 488 goat anti-mouse IgG [H+L], cat #A11029; Life Technologies) in 500-fold dilution in PBS containing 3% BSA. Nuclei were stained for 10 minutes with DAPI (ProLong Gold Antifade Reagent with DAPI, cat #P36931; Life Technologies). After a final wash, cells were mounted in commercial mounting medium (Vectashield; Vector Laboratories,

FIGURE 1. Molecular density of CD40 on Graves' orbital fibroblasts (GOFB) and fibrocytes. (A) Constitutive CD40 display on fibrocytes is exhibited by immunofluorescence (*inset*: negative isotype control). Level of CD40 surface expression, as determined by FACS analysis, is higher on fibrocytes than that on GOFB (MFI fibrocytes, 4.5 ± 0.4 versus GOFB, 2.2 ± 0.2 , $P < 0.005$). (B) CD40 expression is upregulated by IFN- γ (1000 units) after 24 hours of treatment in both fibrocytes and orbital fibroblasts, as shown by flow cytometry. (C) Real-time PCR demonstrates that CD40 mRNA is upregulated by IFN- γ in fibrocytes from healthy donors ($n = 3$, * $P < 0.05$; *** $P < 0.0001$ versus control unstimulated cultures).

Burlingame, CA) and examined with a confocal microscope (Leica SP5 Confocal Microscope; Leica, Wetzlar, Germany).

Western Blot

Anti-AKT, pAKT (Ser473), and β -actin Abs were from Cell Signaling (Boston, MA; cat #9272, #5171, and #3700, respectively). Anti-C-jun antibody was from Santa Cruz Biotechnology (Santa Cruz, CA; cat #sc-45). Nuclear proteins were isolated using the NE-PER nuclear and cytoplasmic extraction kits (cat #78833; Thermo Fisher Scientific, Rockford, IL). Western blots were performed as described previously.²⁹

Flow Cytometry

Cultivated Cells. Levels of CD40 expression on fibrocytes and GOFB were determined by FACS analysis before and after 24-hour treatment with IFN- γ . Cells were washed and removed from the substratum by scraping, stained with PE mouse IgG1, κ isotype control (cat #554680; BD Biosciences), and PE mouse anti-human CD40 (cat #555589; BD Biosciences Pharmingen, San Diego, CA), and subjected to FACS analysis as published previously.²⁴

Peripheral Blood Mononuclear Cells. Quantification of fibrocytes identified directly in peripheral blood was achieved by FACS, which was performed within 24 hours of blood collection as published recently and based on the technique reported by Moeller et al.³⁰ Briefly, 100 μ L whole blood was placed in 12 $\times$ 75-mm polypropylene tubes and 2 mL commercial solution (Pharm Lyse solution 1 $\times$, cat #555899; BD Biosciences) was added for 10 minutes at room temperature. Cells were centrifuged at 500g for 5 minutes and washed. They were resuspended in 100 μ L staining buffer (SB), which was prepared in PBS (Life Technologies) containing 2% FBS (Life Technologies) with 0.1% sodium azide (Sigma-Aldrich). The following anti-human fluorochrome-conjugated MoAbs were added: CD45-PE (cat #347464), CD34-PE (cat #550761), CD14-FITC (cat #555397), CD11b-PE (cat #555388), CD86-FITC (cat #555657),

CD90-FITC (cat #555595), CD40-PE (cat #555589), mouse IgG1, κ isotype control-FITC (cat #555748), mouse IgG1, κ isotype control-PE (cat #554680) from BD Biosciences, and CXCR4-PE (cat #FAB173P) from R&D Systems (Minneapolis, MN). Following a 20-minute incubation in the dark at 4°C, cells were resuspended and washed twice in SB. Cells were then permeabilized with a commercial fixation/permeabilization solution kit (CytoFix/CytoPerm; BD Biosciences) for 20 minutes at 4°C, washed, and resuspended in 100 μ L Perm/Wash buffer (cat #554723; BD Biosciences). Cells were incubated with biotinylated goat anti-human collagen type I polyclonal Ab (cat #AB758B; Millipore, Billerica, MA) for 20 minutes. Rinsed cells were incubated with streptavidin-conjugated FITC (cat #554060; BD Biosciences), and fixed with 1% paraformaldehyde. Analysis was performed using a flow cytometer (FACS Calibur; BD Biosciences). At least 5×10^4 events were collected. Mean fluorescent intensity (MFI) was calculated as a ratio of mean fluorescence sample/isotype fluorescence. Percentage positive expression was defined as the fraction of cells with increased fluorescent intensity compared with isotype control. Fibrocyte quantification was determined as the percentage of monocytes coexpressing CD45, Col1, and CD34 or CD45, Col1, and CD40. Data from 23 patients with TAO are included in Figure 5A and 19 patients with TAO are included in Figure 6. Inadequate sample volume prevented assessment of CD40 expression in fibrocytes from 4 of 23 patients with TAO.

Statistics

Statistical analysis was performed using two-tailed *t*-test or one-way ANOVA with Tukey's multiple comparison post test (as needed). Data are reported as the mean $\pm$ SE.

The data in Figures 5 and 6 do not follow a normal distribution (Shapiro-Wilk goodness-of-fit test value: $P < 0.05$); therefore, group differences were tested with Kolmogorov-Smirnov and Wilcoxon Mann-Whitney *U* tests.³¹ A *t*-test on arcsine square root transformed data, used to normalized percentage data, was also performed.³² These

FIGURE 2. CD40 ligation induces several cytokines in cultivated fibrocytes. Cells were treated with control membrane or CD40L recombinant membrane (100 ng/mL) for 24 hours. Conditioned media were then subjected to Luminex assessment of the cytokines indicated. Results are combined from two experiments (* $P < 0.05$; ** $P < 0.001$; *** $P < 0.0001$ versus control membrane-treated cultures).

tests resulted in statistically significant differences between TAO and healthy groups, with all values of $P < 0.0001$.

RESULTS

IFN- γ Upregulates CD40 on Fibrocytes and Orbital Fibroblasts

GOFB display CD40 when treated with IFN- γ .¹⁶ Fibrocytes express CD40 constitutively, as demonstrated by immunofluorescence and flow cytometry (Fig. 1A). CD40 density on fibrocytes, as measured by MFI, was significantly greater than that seen on GOFB (Fig. 1A). However, the density of CD40 on fibrocytes from donors with TAO and those from healthy subjects was similar (data not shown). Treatment with IFN- γ for 24 hours increased the surface expression of CD40 even further on fibrocytes from both sources (Fig. 1B). A similar enhancement was observed in GOFB, consistent with earlier reports.¹⁶ Figure 1C demonstrates that the steady state CD40 mRNA level reached the highest level of expression 18 hours after treatment.

CD40L Induces IL-6 Expression by Fibrocytes through a Pretranslational Mechanism

Fibrocytes were treated without or with CD40L for 24 hours. Media were then subjected to analysis of cytokine content. Significant induction of several cytokines including IL-6, TNF- α , IL-8, MCP-1, and RANTES (Regulated on Activation, Normal T Cell Expressed and Secreted) could be detected by multiplex analysis (Fig. 2; $P < 0.05$ versus control for all cytokines). These have been implicated in early phase immune responses and in GD and TAO.^{15,24,25,33} The time course of IL-6 mRNA expression reached a peak between 2 and 6 hours (Fig. 3A). The addition of CD40L resulted in a 28-fold increase of IL-6 mRNA ($n = 3$, $P < 0.001$).

Akt and NF- κ B Mediate IL-6 Induction by CD40L

Since Akt and NF- κ B mediate CD40-dependent signaling in other cell types, their potential role in IL-6 induction was investigated in fibrocytes. Inhibiting Akt activity with the

FIGURE 3. CD40 ligation induces IL-6 expression through Akt. (A) IL-6 mRNA expressions in cultivated fibrocytes were followed by real-time PCR after CD40L treatment for 24 hours ($n = 3$, * $P < 0.05$; ** $P < 0.001$ versus control unstimulated cultures). (B) CD40L-initiated activation of IL-6 expression in fibrocytes. (C) CD40L-provoked upregulation of IL-6 in fibrocytes is blocked by Akt inhibition. Cells were pretreated with Akt inhibitor IV (AktI) and then CD40L-containing membranes were added to culture medium for 1 hour. Significant difference was found between cultures treated with control membranes versus CD40L ($P < 0.0001$) and CD40L versus CD40L plus AktI ($P < 0.001$). (C) Akt phosphorylation at Ser473 in normal fibrocytes is increased by CD40 ligation after 30 minutes of incubation, whereas AktI suppresses Akt phosphorylation as shown with Western blot analysis.

specific inhibitor, AktI, substantially blocks the induction of IL-6 by CD40L (Fig. 3B, $n = 3$, $P < 0.001$). Furthermore, CD40L-induced phosphorylation of Akt at serine 473 could be completely attenuated with AktI (Fig. 3C). Similarly specific inhibitors of NF- κ B, including MG132 and PDTC, were found to block the induction (Fig. 4A, $n = 3$, $P < 0.05$). CD40L induced NF- κ B p65 translocation into the fibrocyte nucleus (Fig. 4B). This nuclear localization was detected under immunofluorescence microscopy (Fig. 4C).

FIGURE 4. CD40 signaling in fibrocytes involves activation of NF-κB and p65 nuclear translocation. (A) CD40L-mediated upregulation of IL-6 in normal fibrocytes is blocked by NF-κB inhibition. Cells were pretreated with either MG132 or PDTC for 1 hour and then treated with either control membranes or those containing CD40L. Result shows significant difference between cultures treated with control membrane versus CD40L ($P < 0.0001$); CD40L versus CD40L plus MG132 ($P < 0.0001$); and CD40L versus CD40L plus PDTC ($P < 0.0001$). (B) CD40 ligation increases nuclear NF-κB p65 fibrocytes. Fibrocytes were treated with control membranes or with recombinant CD40L membrane for 1 hour; cytosolic and nuclear proteins were extracted and subjected to Western blot analysis of NF-κB p65. β-Actin and c-jun served as the loading control for cytosolic and nuclear protein, respectively. (C) Immunostaining of cultured fibrocytes shows the translocation of NF-κB p65 from the cytosol to nucleus as a result of CD40L induction. Yellow arrows indicate NF-κB p65 in cytosol of untreated cells and in nucleus of CD40-stimulated fibrocytes.

Increased Circulating CD40⁺ Fibrocyte Frequency In Situ in TAO

Given the potential importance of fibrocytes in immune responses, the relative frequency of peripheral blood CD40⁺ fibrocytes was determined using multiparameter flow cytometry. Fibrocytes are identified at increased frequency in TAO (Fig. 5A, TAO, $19 \pm 4\%$, $n = 23$, versus healthy $2.5 \pm 0.9\%$, $n = 19$, $P < 0.0001$), based on coexpression of CD45, Col1, and

CD34 (Fig. 5B). The vast majority of these cells also exhibited a CD14⁺CD86⁺ phenotype, attesting to their monocyte derivation (Fig. 5B). Consistent with previous reports, these fibrocytes express CXCR4 (Fig. 5B), CD11b, and CD90 (data not shown). Based on these markers, the phenotypes of fibrocytes from patients with TAO appear indistinguishable from those of healthy controls.

The CD45⁺Col1⁺CD40⁺ fibrocytes were significantly more frequent in patients with TAO than that in healthy individuals

FIGURE 5. Circulating fibrocytes are more abundant in TAO in situ. (A) Fibrocytes were identified by FACS analysis of the peripheral blood as CD45⁺Col1⁺CD34⁺ monocytes. These cells were abundant in the PBMC from patients with TAO ($n = 23$) yet rare in healthy controls ($n = 19$) ($34.8 \pm 5.0\%$ vs. $6.5 \pm 2.7\%$, respectively; $P < 0.0001$). (B) The phenotype of these cells was confirmed to include expression of CD45, Col1, CD34, CD14, CD86, and CXCR4 by flow cytometry.

FIGURE 6. Circulating CD40⁺ fibrocytes are more frequent in TAO in situ. The fraction of CD45⁺CD11⁺CD40⁺ fibrocytes is substantially greater in those from donors with TAO ($n = 19$) compared with healthy controls ($n = 19$, $10 \pm 2\%$ vs. $0.6 \pm 0.2\%$, respectively; $P < 0.0001$).

(Fig. 6) (TAO, $10 \pm 2\%$, $n = 19$, versus healthy $1.0 \pm 0.2\%$, $n = 19$; $P < 0.0005$). No correlation could be detected between levels of CD40⁺ fibrocytes and clinical activity score (data not shown).

DISCUSSION

CD40 plays an important role in both immune activation and anergy, depending on the molecular context. In several experimental models of autoimmunity, CD40 activation overcomes peripheral tolerance.^{34–36} In contrast, antigen expression in the absence of activation can lead to peripheral anergy.^{37,38} The importance of CD40 in GD and TAO has been suggested previously from our work^{3,16,18,19,39} and that of others.⁴⁰ Both GOFB and GD thyroid fibroblasts express CD40 and produce proinflammatory cytokines in response to ligation.¹⁵ CD40–CD40L interactions in these cells lead to induction of PGHS-2 and production of PGE₂.^{16,18,39} Additionally, CD40L in GOFB has been shown to induce hyaluronan synthesis.¹⁸ That glycosaminoglycan accumulates in the orbit and infiltrates extraocular muscles in TAO.⁴¹ Here we show for the first time that fibrocytes constitutively express CD40 and produce proinflammatory cytokines such as MCP-1, IL-8, and IL-6 in response to CD40L. The similarities in CD40 expression and function on GOFB and circulating fibrocytes further support our hypothesis that the latter infiltrate the orbit²⁴ and thyroid¹⁵ in GD, where they might provide antigen-specific T-cell stimulation.^{9,42}

CD40⁺ fibrocytes are considerably more frequent in patients with TAO. In culture, constitutive levels of CD40 are higher on fibrocytes than those found on GOFB (Fig. 1A). IFN- γ can induce CD40 expression in both cell types (Fig. 1B) and can be detected in orbital tissues from patients with TAO.¹⁶ Moreover, this pathway may mediate inflammation and fibrosis.⁴³

FIGURE 7. Schematic view of CD40 signaling pathway of IL-6 production in fibrocytes. CD40–CD40L interaction activates a downstream signaling pathway, resulting in the production of proinflammatory cytokine, IL-6. CD40 signaling for IL-6 is mediated by Akt phosphorylation and subsequent NF- κ B translocation.

Activation of CD40 occurs through its engagement with CD40L, which is expressed on T cells and fibroblasts.^{44,45} This in turn results in the production of IL-6, TNF- α , IL-8, and RANTES. IL-6 has been implicated in the pathogenesis of autoimmune disease⁴⁶ and specifically GD, where levels have been observed.^{47,48} This cytokine has been detected in thyroid tissues, extraocular muscles, and orbital fat from these patients.^{49,50}

CD40 can activate several signaling pathways, including phosphatidylinositol 3-kinase. These signaling events culminate in recruitment of NF- κ B to the cell nucleus.⁵¹ In autoimmune inflammatory bowel disease, human colonic fibroblasts respond to CD40L by producing IL-8, IL-6, and MCP-1 in a process mediated by NF- κ B.⁵² Genetic analysis has linked CD40 and NF- κ B signaling in rheumatoid arthritis, suggesting the central role of both molecules.⁵³

Our current findings strongly suggest that CD40 and its postregulatory signaling may play significant regulatory roles in human fibrocytes (Fig. 7). Since these cells appear to participate in the pathogenesis of TAO, it is possible that this pathway could represent an attractive therapeutic target for orbital Graves' disease, which remains a vexing manifestation of autoimmunity.⁵⁴

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