Orbital Fibroblasts from Patients with Thyroid-Associated Ophthalmopathy Overexpress CD40: CD154 Hyperinduces IL-6, IL-8, and MCP-1

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Purpose. Fibroblast diversity represents an emerging concept critical to our understanding of tissue inflammation, repair, and remodeling. Orbital fibroblasts heterogeneously display Thy-1 and exhibit unique phenotypic attributes that may explain the susceptibility of the human orbit to thyroid-associated ophthalmopathy (TAO). In the present study the authors investigated the role of CD40 ligation on macrophage chemoattractant protein-1 (MCP-1), IL-6, and IL-8 expression in fibroblasts from patients with TAO.

Methods. Human orbital fibroblasts were cultured from tissues obtained with informed consent from patients with TAO and from patients undergoing surgery for other noninflammatory conditions. The fibroblasts were then examined by flow cytometry, microscopy, and cytokine assays.

Results. The authors report that orbital fibroblasts from patients with TAO expressed elevated levels of CD40. Surface CD40 could be further upregulated by IFN-γ in TAO and control fibroblasts. This upregulation was mediated through Jak2 and could be blocked by dexamethasone and AG490, a powerful and specific inhibitor of tyrosine kinase. Treatment with CD154, the ligand for CD40, upregulated the expression of IL-6, IL-8, and MCP-1 in TAO fibroblasts but failed to do so in control cultures. Thy-1$^+$ fibroblasts displayed higher CD40 levels than did their Thy-1$^-$ counterparts and were largely responsible for this cytokine production. IL-1β also induced MCP-1, IL-6, and IL-8 more vigorously in TAO-derived fibroblasts.

Conclusions. Characterization of orbital fibroblasts and their differential expression of cytokines and receptors should prove invaluable in understanding the site-specific nature of TAO and the development of specific therapies. (*Invest Ophthalmol Vis Sci.* 2009;50:2262–2268) DOI:10.1167/iovs.08-2328

Divergent phenotypic attributes of fibroblasts may help explain tissue-specific functions and anatomic site-selective vulnerability to disease. Fibroblast diversity represents an emerging concept potentially critical to our understanding of tissue inflammation, repair, and remodeling. This is true of systemic disease directed at the orbit, such as that occurring in Graves' disease (GD) and its orbital component, thyroid-associated ophthalmopathy (TAO). In TAO, lymphocytes, monocytes, and mast cells infiltrate orbital tissues, which become inflamed and extensively remodeled.[1] Why mononuclear cells are trafficked to the orbit remains uncertain, but targeting these tissues may result from their unique immunologic properties. Physical peculiarities such as the bony orbit and its unique pattern of blood flow and lymphatic drainage might influence disease distribution.[2,3] In addition to inflammation, the pathology of TAO involves accumulation of the nonsulfated glycosaminoglycan hyaluronan,[4] fibrosis, and increased fat volume.[5] The active phase of TAO is most frequently self-limited and can culminate in diminished eye motility,[6] orbital congestion, and compressive neuropathy.[5] The potentially complex interplay between mononuclear cells and orbital fibroblasts may underlie the unusual tissue reactivity and remodeling that occurs in TAO.

Orbital fibroblasts represent a heterogeneous population based on the cell-surface display of Thy-1, a membrane-associated glycoprotein.[7] When sorted into subsets, Thy-1$^+$ fibroblasts express divergent cytokine profiles compared with their Thy-1$^-$ counterparts after activation by proinflammatory molecules such as IL-1β and CD154, the cognate ligand of CD40.[8,9] These subsets also have distinct potential for terminal cell differentiation.[7] Thus, each fibroblast subset may serve distinct roles in health and disease.

CD40 and its cognate ligand CD154 represent an important activational pathway initially implicated in T-cell/B-cell interactions but more recently found important in communication between many cell types, including endothelial cells, smooth muscle cells, fibroblasts, bone marrow-derived cells, and follicular dendritic cells.[10,11] CD40 is a member of the TNF-α receptor superfamily, which uses phosphorylation of TRAFS and NFκB for cell signaling.[12,13] The interaction of CD40 on B cells with CD154-displaying activated T cells provides a costimulatory signal that induces T-cell–dependent B-cell proliferation and differentiation and leads to antibody production.[14] Professional antigen-presenting cells such as macrophages and dendritic cells require CD40 signaling for activation and use CD40 as a cochaperone-like receptor, mediating the uptake of exogenous hsp70-peptide complexes.[10] Moreover, CD154 can act as a soluble cytokine.[15] Aberrant CD40-CD154 interactions appear to corrupt humoral immunity in autoimmune diseases

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such as systemic lupus erythematosus and rheumatoid arthritis.[15]

In the present study, we assessed several potentially important inflammatory attributes of orbital fibroblasts and their use of the CD40/CD154 molecular bridge. Previously, we found that many of the actions of CD154 in orbital fibroblasts are mediated through the intermediate induction of IL-1α, a cytokine that induces several cytokines and hyaluronan expression by orbital fibroblasts.[16,17] These include the induction of prostaglandin endoperoxide H synthase-2 (PGHS-2) gene expression.[18] In the present study, we investigated the impact of CD154 on macrophage chemoattractant protein-1 (MCP-1), IL-6, and IL-8 expression in fibroblasts from patients with TAO. These findings help define more clearly the potential roles of fibroblast diversity in tissue reactivity and remodeling found in TAO.

Materials and Methods

Materials

Anti–Thy-1 and anti–CD40 antibodies were purchased from BD Biosciences/PharMingen (San Diego, CA). Rh IFN-γ and IL-1β were obtained from BioSource (Camarillo, CA). AG490 was supplied by Calbiochem, and dexamethasone was supplied by Sigma-Aldrich (St. Louis, MO). Culture medium and fetal bovine serum (FBS) were supplied by Gibco-Invitrogen (Carlsbad, CA). Recombinant CD154-expressing membranes and their controls were prepared as described previously.[19]

Cell Culture

Human orbital fibroblasts were cultivated as reported previously.[20] Tissue explants were obtained from patients undergoing orbital decompression for severe TAO or surgery for a noninflammatory condition. These activities were undertaken after informed consent was obtained from the donors according to procedures approved by the Institutional Review Boards of the Harbor-UCLA Medical Center/Los Angeles Biomedical Institute, Center for Health Sciences at UCLA, and the tenets of the Declaration of Helsinki. Ten fibroblast strains from patients with stable TAO and 4 control fibroblast strains were examined. Donors were euthyroid at the time of donation. Tissue explants attached to plastic culture dishes and were covered with medium containing glutamine and fetal bovine serum (FBS, 10%). They were incubated in a 37°C humidified incubator with a 5% CO_2 environment. Resulting fibroblast monolayers were serially passaged with gentle trypsin/EDTA treatment and were used for studies between the second and twelfth passages from culture initiation. Cultures were free of cells expressing factor VIII, α-smooth muscle-specific actin, and cytokeratin.[21]

Preparation of Thy-1$^+$ and Thy-1$^-$ Subsets

Separation of fibroblasts on the basis of Thy-1 display was conducted as reported previously.[20] Briefly, nearly pure Thy-1$^+$ and Thy-1$^-$ subsets were generated after three or four rounds of magnetic bead selection.[22,23] Subsets exhibited a stable Thy-1 phenotype in culture, as monitored by flow cytometry, and were either greater than 99% Thy-1$^+$ or greater than 97% Thy-1$^-$.

Methods for Immunostaining

Histologic sections from formalin-fixed, paraffin-embedded tissue blocks were subjected to heat-induced epitope retrieval using a steamer at 95°C for 25 minutes in a 0.01 M citrate buffer, pH 6.0, for CD40, CD45, and smooth muscle actin. For factor VIII staining, slides were pretreated with proteinase K for 15 minutes at 37°C (Dako, Carpinteria, CA). Sections were incubated with mouse monoclonal antibody against CD40 (Novocastra, Bannockburn, IL), or CD45 (Dako), respectively. Staining for smooth muscle actin was performed with rabbit anti–α-smooth muscle actin (Abcam Inc., Cambridge, MA), and factor VIII staining was performed with rabbit anti–von Willebrand factor (Dako). After incubation with primary monoclonal antibody or polyclonal antiserum, localization of antigen was performed with a staining kit (EnVision+ System-HRP; DakoCytomation, Carpinteria, CA). After the diaminobenzidine reaction, slides were counterstained in hematoxylin. Positive controls included tissues with known specificity for the antibodies (colon, tonsil) and for vessels within the tissue, which served as positive controls for factor VIII, smooth muscle actin, and CD45. Negative controls consisted of substitution of the primary antibody with isotype-specific non–cross-reacting monoclonal antibody (for the monoclonal antibody reagents) and species-specific nonimmune serum for the polyclonal antisera.

Flow Cytometry

These techniques have been published.[7] Briefly, 1×10^6 cells were placed in 12 × 75-mm polypropylene tubes, and fluorochrome-conjugated monoclonal antibodies (1 μg/10^6 cells) were incubated in the dark for 20 minutes at room temperature. Cells were washed twice with staining buffer (SB; phosphate-buffered saline and 4% fetal calf serum), resuspended, and maintained at 4°C until cytometric analysis (within 24 hours) using a flow cytometer (FACSCalibur; BD Biosciences). Mean fluorescence intensity (MFI) was calculated as a ratio of mean fluorescence sample/isotype fluorescence.

Cytokine Assays

Fibroblasts were cultured to confluence and treated for 48 hours with or without RhIFN-γ (100 U/mL) in MEM with 0.1% FBS, followed by treatment with IL-1β (10 ng/mL), CD154 membranes, or control membranes for 48 hours. Previous work in our laboratory and that of others demonstrated these conditions provide maximal fibroblast response to these molecules.[9,24,25] Culture medium was analyzed for IL-6 and IL-8 content with the use of bead-based assays (Luminex; Millipore, Billerica, MA). MCP-1 content was analyzed by standard sandwich ELISA (R&D Systems, Minneapolis, MN).

Statistical Analysis

Values are reported as mean ± SE. Statistical analysis was performed using a two-tailed Student's *t*-test with a confidence level greater than 95%.

Results

TAO Orbital Fibroblasts Express CD40

Orbital fibroblasts from patients with TAO produce PGE_2 and hyaluronan in response to CD40 ligation in culture.[17] Thus, we examined whether CD40 staining differed in tissues from patients with and without TAO. CD40$^+$ cells (arrows) were more abundant in disease-derived tissues (Figs. 1B–D) than in controls (Fig. 1A). CD40$^+$ staining was present in fibroblast-shaped cells and areas predominated by fat (Figs. 1B–D). Infiltrating mononuclear cells and vascular structures also express CD40. However, sections stained with the leukocyte-specific marker CD45 detected few infiltrating leukocytes (Fig. 1E), and factor VIII, which stains endothelial cells, was restricted to vessels (Fig. 1F). CD40$^+$ cells were detected in areas devoid of factor VIII and CD45 staining and were morphologically typical of fibroblasts.

We next examined CD40 displayed on isolated fibroblasts by flow cytometry (Fig. 2, upper panel). The MFI of CD40 expression was 3.1 ± 0.3-fold ($n = 7$) in disease-derived cells, whereas that from controls was 1.1 ± 0.1-fold ($n = 4$; $P < 0.001$) compared with isotype control. Treatment with IFN-γ (100 U/mL) for 72 hours resulted in a dramatic upregulation of CD40 in fibroblasts from both sources (MFI multiple 8.0 TAO, 6.4 control). Thus, CD40$^+$ fibroblasts were abundant in the

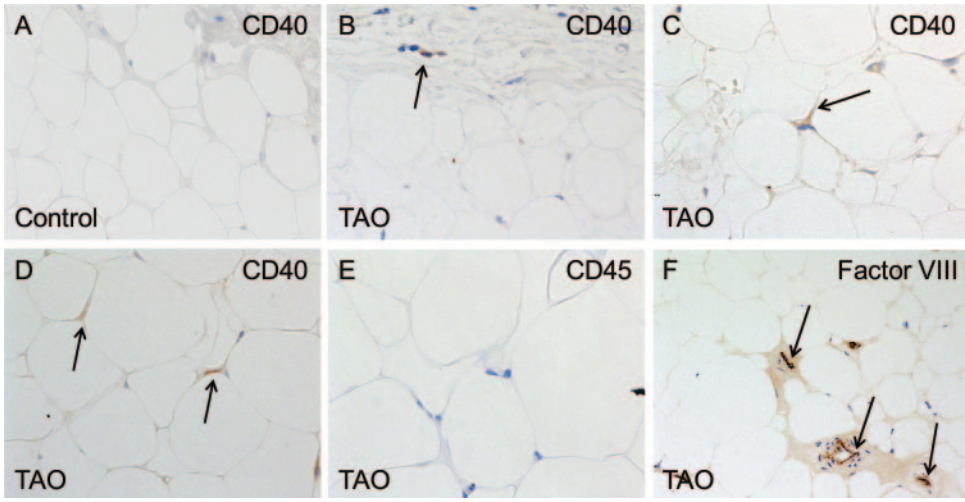

FIGURE 1. Immunostaining of CD40 in control orbital tissue and TAO. Fibroblast-like $CD40^+$ cells (*arrows*) are more numerous in the stromal and fat predominant areas of thin sections from a patient with TAO (**B–D**) than in control tissue (**A**). (**E**) Minimal expression of CD45 in sections from disease-derived tissue. (**F**) Factor VIII staining is restricted to vascular endothelium.

orbital connective tissue of patients with TAO, and the receptor density on cells in vitro was considerably greater than that found on controls. This divergence in receptor density suggests that TAO fibroblasts may interact with CD154-bearing T cells in a disease-specific manner. Given that IFN- γ has been detected in active TAO, we assessed the mechanism through

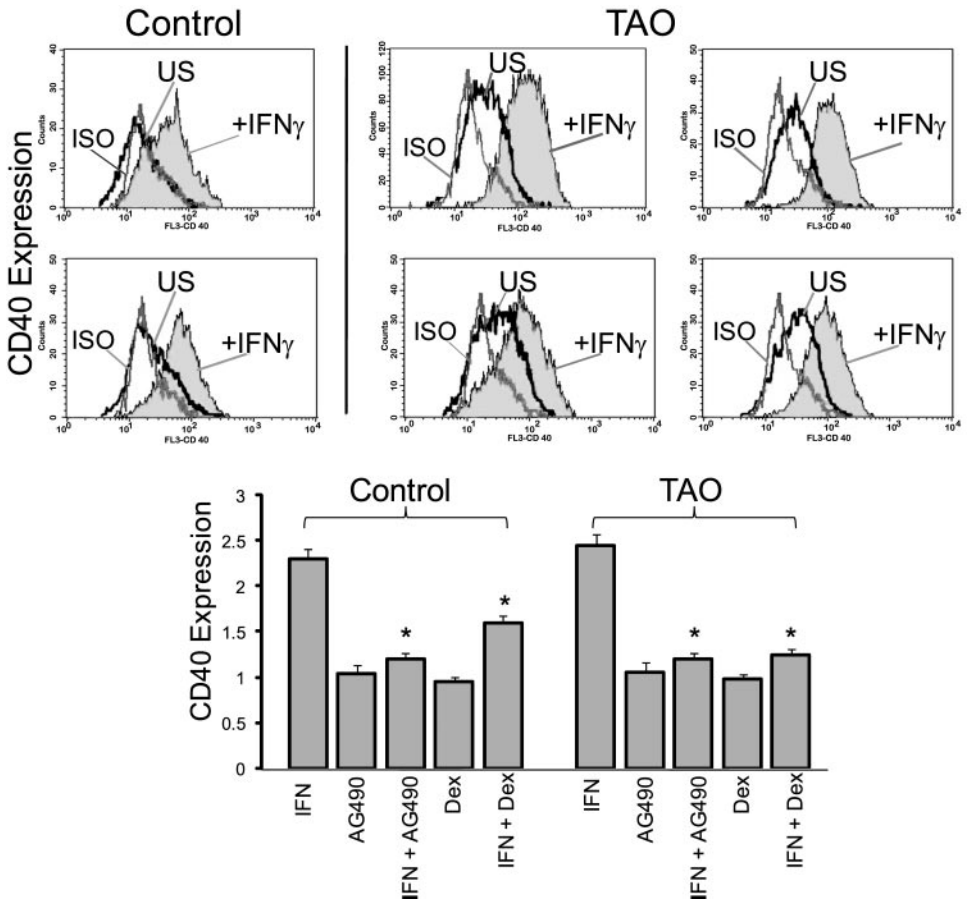

FIGURE 2. CD40 displayed by orbital fibroblasts and its upregulation with IFN-γ. *Upper*: TAO and control fibroblasts were cultured. They were stimulated with or without IFN-γ (100 U/mL) for 48 hours and stained with anti–CD40 or its isotype (*dark gray open histogram*) antibodies. *Lower*: fibroblasts were incubated with IFN-γ with or without AG490 (75 μM) or dexamethasone (10 nM) for 72 hours. CD40 expression is presented as the multiple of constitutive expression.

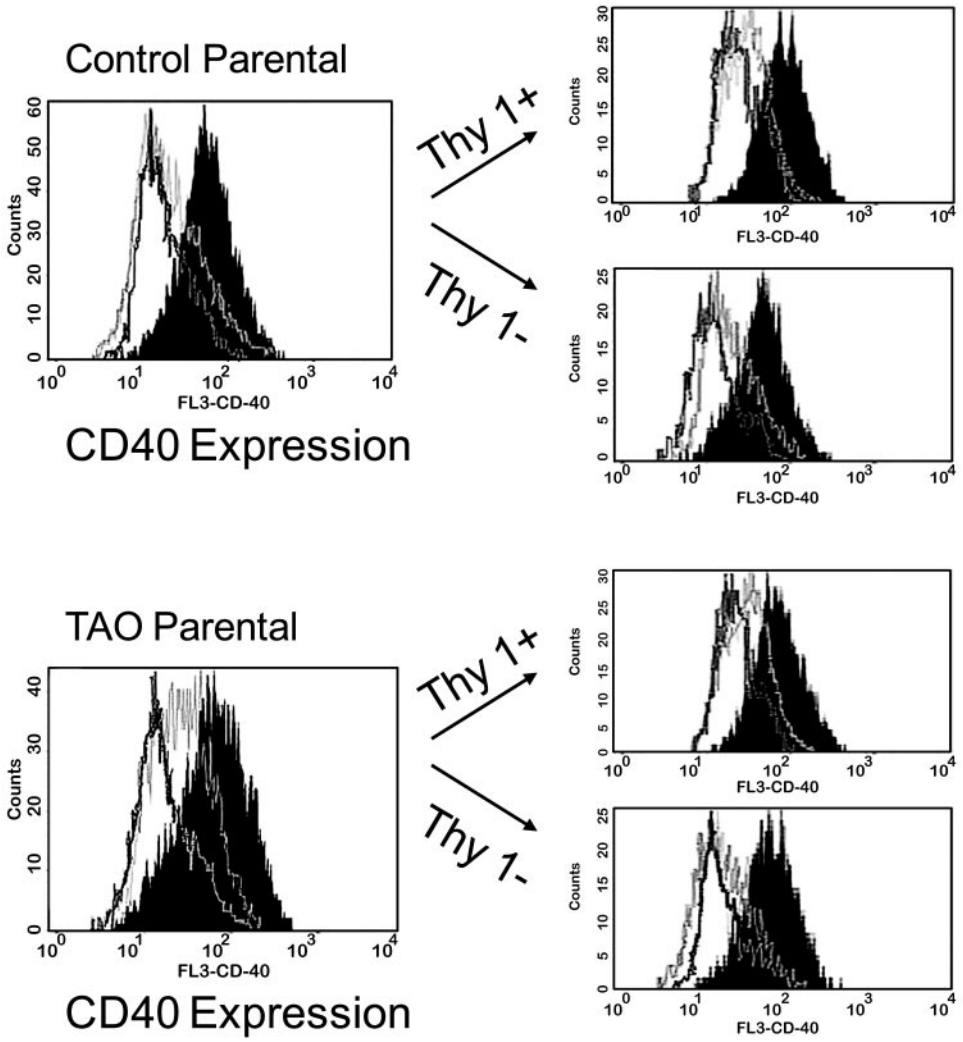

FIGURE 3. Thy-1$^+$ fibroblasts from patients with TAO express CD40. Parental fibroblast strains were sorted based on Thy-1 display and cultured. Subsets were then stained for CD40 expression. Unstimulated fibroblasts were stained with an isotype-control antibody (*dark gray open histogram*) or anti–CD40 (*light gray open histogram*), whereas others were treated with IFN-γ (100 U/mL) for 48 hours and stained with anti–CD40 antibodies (*black histogram*).

which it upregulates CD40.[26,27] We focused on Jak2 because that kinase plays a central role in signaling found in TAO fibroblasts.[18] In the presence of AG490 (75 μM, a specific inhibitor of Jak2), IFN-γ–dependent CD40 expression by TAO and control fibroblasts was markedly reduced to levels observed in the absence of cytokine (Fig. 2, lower panel). Treatment with dexamethasone (10 nM) also inhibited the IFN-γ induction of CD40. Thus, interfering with Jak2 signaling appears to markedly attenuate CD40 expression induced by IFN-γ in TAO and control fibroblasts. On the other hand, the elevated basal CD40 levels found in TAO fibroblasts were unaffected by AG490 and dexamethasone, suggesting that Jak2 does not support the elevated constitutive receptor display found in TAO (Fig. 2, lower panel).

We next assessed whether high-level CD40 expression was global and distributed widely among fibroblast subsets segregated on the basis Thy-1 display or confined to a particular subset. CD40 levels in mixed populations were compared with those of sorted fibroblast subsets and were found to be highly expressed by parental TAO fibroblasts (MFI, 3.2 ± 0.3-fold greater than isotype) compared with their control counterparts (MFI, 1.1 ± 0.2-fold greater than isotype; Fig. 3). CD40 was minimally expressed in the Thy-1$^+$ and Thy-1$^-$ control populations after sorting and culture (Fig. 3, upper panel). However, its expression in TAO fibroblasts was found predominantly in the Thy-1$^+$ subset (Thy-1$^+$ MFI, 3.4 ± 0.3-fold; Thy-1$^-$, MFI 1.4 ± 0.3-fold; $P < 0.05$; $n = 4$).

CD154 Induces IL-6, IL-8, and MCP-1

We determined the functional consequences of CD40 display by incubating control and TAO cultures with CD154-expressing insect membranes or control membranes and assessed IL-6 and IL-8 production resulting from each treatment. The parental strain, consisting of 55% Thy-1$^+$ and 45% Thy-1$^-$ fibroblasts, and its derivative subsets were stimulated with CD154 for 48 hours. Figure 4 demonstrates IL-6 and IL-8 overproduction in parental TAO strains compared with control cultures after CD154 treatment (representative of four replicates; TAO vs. control; $P < 0.01$). The TAO Thy-1$^+$ subset also exhibited substantial IL-6 and IL-8 production, whereas production in Thy-1$^-$ fibroblasts was considerably lower ($P < 0.01$). Thus, TAO-derived Thy-1$^+$ cells appear more responsive to CD154 than do their Thy-1$^-$ counterparts with regard to IL-6 and IL-8

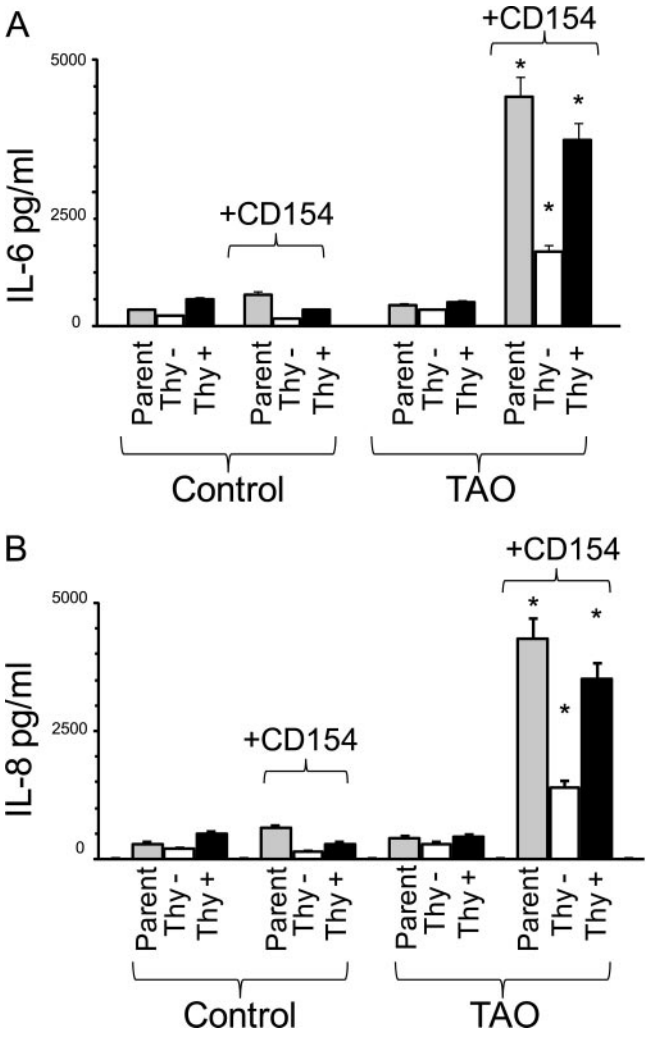

FIGURE 4. CD154 induces IL-6 and IL-8 in Thy-1$^+$ TAO orbital fibroblasts. Fibroblasts from donors with TAO and those without disease were isolated, cultured, and treated with IFN-γ for 48 hours followed by incubation with CD154 membranes for 48 hours. Concentrations of IL-6 (**A**) and IL-8 (**B**) were determined in parental, Thy1$^+$, and Thy1$^-$ subsets. $^*P < 0.01$, representative of four experiments.

expression. In contrast, control fibroblasts produced low IL-6 and IL-8 levels under these same conditions. Thus, the higher levels of CD40 found in Thy-1$^+$ TAO fibroblasts may be critical to the generation of IL-6 and IL-8 in response to CD154. Given the role of immune cell infiltration in this disease, we also examined the expression of MCP-1. Control fibroblasts expressed modest MCP-1 under basal culture conditions, and the levels were not induced by CD154 (Fig. 5). In contrast, the TAO parental strain and both sorted subsets expressed increased levels of MCP-1 in response to CD154 ($n = 4$; $P < 0.01$). Thus, in contrast to IL-6 and IL-8 expression, the magnitude of MCP-1 induction was not proportional to CD40 expression levels in Thy-1$^+$ and Thy-1$^-$ TAO subsets.

IL-1β Induces IL-6 and IL-8 Production in TAO Fibroblasts

Given the divergent phenotypic and functional properties of TAO orbital fibroblasts with regard to the CD40/CD154 bridge, we explored whether responses of these cells might be generalized to other cytokines important in TAO. IL-1β appears to have a central role in TAO by inducing cytokine and extracellular matrix production in a site-selective manner.[16–18] Moreover, IL-1α is induced by CD154 in TAO fibroblasts and serves as a critical intermediate for the induction of PGHS-2.[24] As shown in Figure 6, several strains of TAO fibroblasts express considerably more IL-8 in response to IL-1β than do control cultures. However, TAO and control fibroblasts produced similar levels of IL-6. Thus, the exaggerated production of proinflammatory cytokine production in orbital fibroblasts in response to other cytokines appears to be cell type and agent specific.

DISCUSSION

CD40 and its ligand, CD154, represent an important cell activation pathway.[28] We have previously reported that CD40 activation in orbital fibroblasts results in the induction of PGHS-2 and the production of PGE_2 and hyaluronan.[24,29,30] Here we demonstrate that unprovoked orbital fibroblasts from patients with TAO express substantially higher levels of CD40 than control fibroblasts. When treated with IFN-γ, CD40 levels are substantially increased in fibroblasts from both sources, an action mediated by Jak2.

We demonstrated that TAO orbital fibroblasts produce significantly more IL-6, IL-8, and MCP-1 through CD40 ligation than do orbital fibroblasts of control donors. Elevated serum IL-8 levels were found previously in hyperthyroid patients with GD, and these may reflect production in sites other than the thyroid.[31] In this study, we demonstrated that CD40/CD154 and IL-1β may enhance IL-8 production in the orbit. High serum IL-6 levels are also associated with GD, and IL-1β increases production of this cytokine uniquely in orbital fibroblasts.[32–34] Local levels in the orbit may promote lymphocyte activation or recruitment.[35] IL-6 induces a number of B-cell genes, and its actions are generally associated with antiapoptotic effects and immunoglobulin production. Moreover, it drives the synthesis of immunoglobulins and is necessary for the normal development of plasma cells.[36] Thus, it remains possible that B cells in the orbit might overproduce immuno-

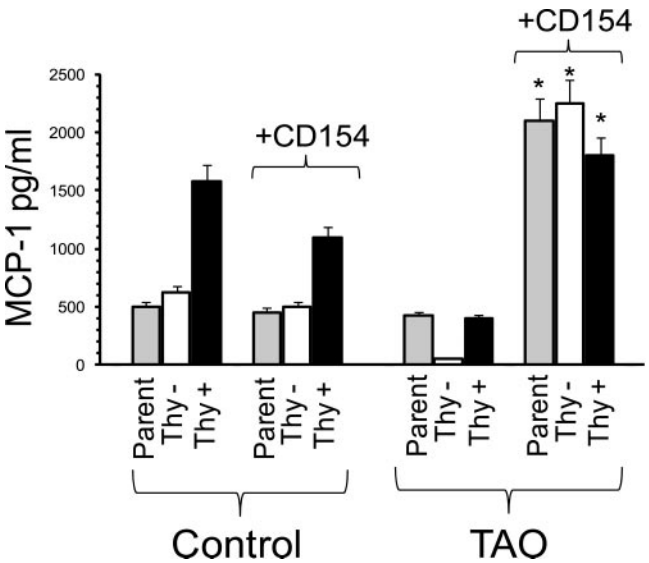

FIGURE 5. CD154 induces MCP-1 in Thy-1$^+$ and Thy-1$^-$ orbital fibroblasts from donors with TAO. Cultures were isolated and treated for 48 hours with IFN-γ and then with CD154 or control membranes for an additional 48 hours. MCP-1 concentrations were determined in parental strains and those sorted into Thy-1$^+$ and Thy-1$^-$ subsets. $^*P < 0.01$, representative of four experiments.

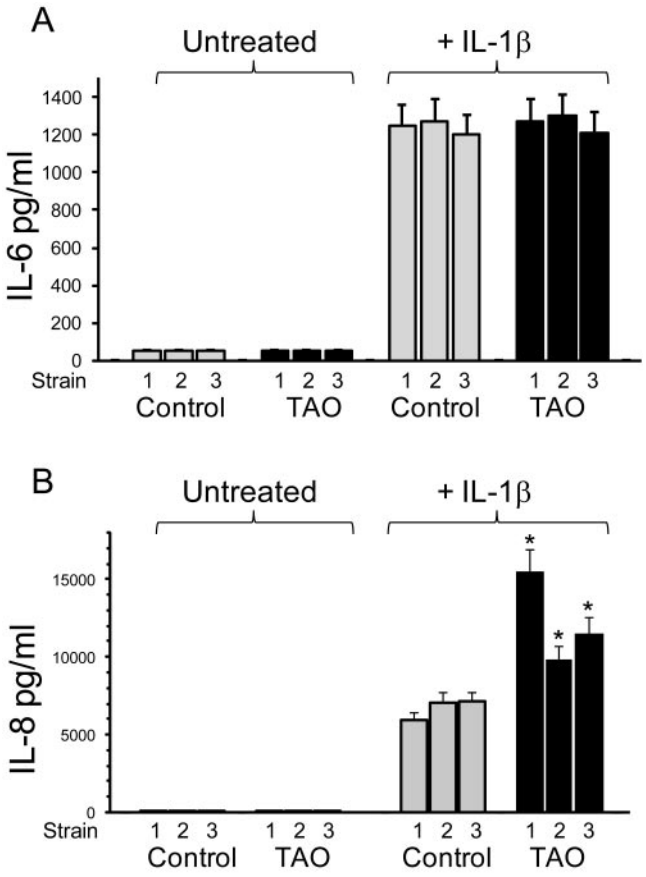

FIGURE 6. IL-1β induces IL-6 and IL-8 in orbital fibroblasts. Fibroblasts from donors with TAO and controls were stimulated with IL-1β (10 ng/mL) for 48 hours. Concentrations of IL-6 (**A**) and IL-8 (**B**) were determined. $^*P < 0.01$, representative of four experiments.

globulins, such as those associated with GD, as a consequence of the high IL-6 levels generated by fibroblasts. With regard to T cells, IL-6 promotes IL-4 synthesis and Th2 development through transcriptional activation of NFAT.[37,38] CD40 interactions with T cells may provide a Th2-type microenvironment. It may also directly promote T-cell migration, effects that are mediated through MAPK, PI3K, and the Jak/STAT pathways.[39] Thus, our findings here that activation of the CD40/CD154 pathway also induces IL-6 and IL-8 suggest that the activation of multiple pathways might underlie the characteristic pattern of inflammatory responses seen in TAO.

MCP-1 is a powerful chemoattractant that targets mononuclear infiltration and promotes inflammation.[31,40] Moreover, it has been implicated previously in autoimmune disease in humans.[41] Macrophage infiltration is a characteristic feature of orbital fat reactivity in active TAO and localizes around blood vessels and between mature adipocytes.[31] MCP-1 mRNA is more abundant in TAO orbital fat, though protein levels in vivo are difficult to quantify.[31] We were unable to detect MCP-1 protein in tissue preparations, but this may reflect the relatively late stage of disease progression in which these samples were obtained because macrophage infiltration was also minimal. Previous reports have demonstrated that IL-1β can induce MCP-1 in a variety of cell types, effects that can be blocked by dexamethasone.[42,43] Here we demonstrate that MCP-1 is also induced in response to CD40 ligation specifically in TAO orbital fibroblasts. Thus, the cytokine may play an important role in macrophage recruitment in the early stages of disease.

Insinuating Jak2 into the induction of CD40 by IFN-γ represents a new insight. We have recently demonstrated the importance of Jak2 in the modulation of PGHS-2 induction by IL-1β in orbital fibroblasts from TAO patients.[18] Coordinate induction of PGHS-2 and PGE_2 in these cells, when treated with proinflammatory cytokines such as CD154 and IL-1β, may contribute to site-specific inflammation. Our finding demonstrating the role of Jak2 in INF-γ–dependent CD40 expression represents a potentially important component of the inflammatory phenotype of these cells.

Features associated with GD and TAO suggest that peculiarities in fibroblast phenotype may underlie susceptibility to tissue remodeling. This possibility has prompted a number of studies examining the cellular characteristics of orbital fibroblasts.[24,44] The relatively high-density surface display of CD40 by TAO orbital fibroblasts suggests that they might interact efficiently with recruited immunocompetent cells such as T lymphocytes. We postulate that their enhanced ability to cross-talk with these cells should promote orbital tissue activation. We have previously demonstrated several unique functional attributes of orbital fibroblasts derived from patients with TAO. These include the exaggerated upregulation of hyaluronan, plasminogen activator inhibitor 1,[35,45] tissue inhibitor of metalloproteinase,[16] PGHS-2,[18] UDP glucose dehydrogenase,[4] and 15-lipoxygenase-1.[46] These findings, all made in vitro using cells derived from primary human tissue, must be interpreted with caution because no animal models of TAO exist. Despite these limitations, our current findings identify features that may prove common to the regulation of these and many other genes. Thus, they hold the potential in aggregate for explaining the tissue specificity of TAO and identifying a cellular feature that might be exploited for therapeutic development, such as the signaling associated with the Jak2 pathway.

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