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Synergy Between CD40 Ligation and IL-4 on Fibroblast Proliferation Involves IL-4 Receptor Signaling

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Fibrosis can be an undesired consequence of activated cellular immune responses. The purpose of this work was to determine whether CD40 ligation and the pro-fibrotic cytokine IL-4 interact in regulating fibroblast proliferation and collagen production, and, if so, the mechanisms used. This study found that the combination of IL-4 and ligation of CD40 on the fibroblast cell surface had synergistic effects in stimulating fibroblast proliferation. In contrast, CD40 ligation negated the inhibitory effects of IFN-γ on fibroblast proliferation. Western blotting analyses of fibroblast crude lysates revealed that a potential mechanism of the synergy between CD40 ligation and IL-4 was the phosphorylation of proteins at 130 kDa and, to a lesser degree, at 95, 85, and 75 kDa. Immunoprecipitation-Western blotting experiments showed that phosphorylation levels of IL-4Rα, Janus kinase 1, insulin receptor substrate 1, and insulin receptor substrate 2, factors with molecular mass close to the observed 130 kDa major phosphorylation band, increased in response to the combined CD40 ligation and IL-4 action. In contrast, there was no evidence that synergy was mediated by an increased expression of IL-4Rα chain, CD40, or the autocrine profibrotic cytokines IL-6 and TGF-β. These findings suggest that CD40-CD40 ligand contacts between fibroblasts and cells secreting IL-4 may promote the profibrotic effects of IL-4 by affecting signal transduction and reducing the anti-fibrotic effects of IFN-γ.


Unwanted tissue damage can occur when fibrosis follows an activated cellular immune response in that tissue. Activated T cells of both Th1 and Th2 types can interact with fibroblasts and regulate their activities by secreting IFN-γ and IL-4, respectively. Mast cells also regulate activities of fibroblasts through IL-4 secretion (1, 2), as do basophils (3) and eosinophils (4). IL-4 is a profibrotic cytokine. It stimulates fibroblast chemotaxis, proliferation, expression of adhesion molecules ICAM-1 and VCAM-1, and production of IL-6 and extracellular matrix proteins in vitro (1, 5–12). Exposure to IL-4 leads to an increase in collagen production in the lungs (4, 13), and correlates with the amount of hepatic fibrosis (14). Normal wound healing is accelerated by IL-4 (2, 15). Treatment with anti-IL-4 and the targeted mutation of IL-4Rα prevent dermal fibrosis in the tight-skin mouse model of scleroderma (16, 17). The profibrotic effects of IL-4 on fibroblasts can be counterbalanced by IFN-γ, which inhibits fibroblast proliferation and production of collagen in vitro (18–21), inhibits experimental renal fibrosis (22), and reduces size of keloids (23). In a bleomycin model of lung fibrosis, inhibition of IL-4 production by lung T cells in the setting of unchanged IFN-γ production leads to reduced lung fibrosis and prolonged survival (24). Conversely, a shift in the balance between IL-4 and IFN-γ mRNA production toward IL-4 in the lungs of scleroderma patients is associated with progressive interstitial lung disease (25).

Activated T cells express CD40 ligand (CD40L) (26), as do mast cells (27), basophils (28), and eosinophils (29). CD40L binds CD40, a member of the TNFR family that is expressed and functionally active in a variety of cell types (30, 31). Fibroblasts from the skin, lung, gingiva, synovium, and spleen all express CD40 (31), which implies potential interactions with T cells through CD40L-CD40 contacts. In fibroblasts, CD40 ligation stimulates proliferation (32) and has other activating effects, including mobilization of NF-κB, production of IL-6 and IL-8 (30, 32, 33), and stimulation of ICAM-1 and VCAM-1 expression (32). CD40 ligation is important in the development of pulmonary fibrosis in animals. Blockage of CD40L-CD40 interactions protects against radiation-induced pulmonary fibrosis (34) and hapten immune pulmonary interstitial fibrosis (35).

The two factors, CD40 ligation and IL-4, interact synergistically in stimulating proliferation (36) and isotype switching, particularly IgE production (37, 38), in B cells. Recently, synergy of the two factors has been described in up-regulation of adhesion molecules expressed on endothelial cells (39). The goal of this work was to investigate whether these two profibrotic factors have synergistic effect on fibroblasts.

Materials and Methods

Fibroblast cell lines

Already existing normal human skin fibroblast lines were used. These lines were established from primary dermal explants as described previously (40). Fibroblast lines were maintained in T75 culture flasks in humidified atmosphere of 5% CO2 at 37°C in high serum tissue culture medium, which was DMEM supplemented with 2 mM glutamine, 2 mM sodium pyruvate, 50 mg/L gentamicin, and 10% bovine calf serum (all from Life Technologies, Grand Island, NY).

Abbreviations used in this paper: CD40L, CD40 ligand; TRAF, TNFR-associated factor; JAK, Janus kinase; IRS, insulin receptor substrate.
Cell proliferation and collagen production assays

Fibroblast cell lines were tested in passages three to seven. They were grown to confluency, detached by trypsinization (Life Technologies), washed, and replated in high serum tissue culture medium in the desired test wells overnight. Two different proliferation assays were used. For use in the CellTiterAQ assay, 96 Non-Radioactive Cell Proliferation Assay (Promega, Madison, WI), fibroblasts were plated at 2 × 10^4 cells per well in 96-well flat-bottom tissue culture plates (Costar, Cambridge, MA) in 0.2-ml cultures. For testing in a [3H]thymidine ([3H]Tdr) proliferation assay, fibroblasts were plated at 3 × 10^4 per well in 12-well plates (Costar) in 2-ml cultures. After overnight incubation in high serum tissue culture medium, the medium in each well was replaced with DMEM containing the same supplements listed above, except that the serum concentration was decreased to 0.5% (low serum tissue culture medium). The fibroblasts were incubated for another 24 h before adding the desired test substances. Recombinant human IL-4, IFN-γ (both from Life Technologies), soluble CD40L (ALEXIS Biochemicals, San Diego, CA), and mouse monoclonal anti-human CD40 (BD Pharmingen, San Diego, CA) were used, alone or in combination. Low serum tissue culture medium alone was the negative control. The CellTiter proliferation assay was done according to the manufacturer’s instructions, after the fibroblasts were incubated with test substances for 3–7 days. Data were expressed as mean OD_{490} ± SD of quadsiplicate cultures. In the [3H]thymidine incorporation assay, fibroblasts were incubated with or without test substances for 2–5 days, then [3H]thymidine was added to each culture to a final concentration of 1 μCi/ml for 8 h of incubation. Then, the cultures were washed with PBS, five times with 5% TCA, and then washed twice with 5% TCA, and lysed in 0.1 N NaOH/0.1% SDS. A 100-μl aliquot of each lysate was measured in a Beckman beta scintillation counter (Beckman Coulter, Fullerton, CA). Data were expressed as cpm ± SD of quadsiplicate cultures.

For the collagen production assay, fibroblasts were plated at 2 × 10^5 cells/well in 6-well plates (Costar), incubated overnight in 3 ml/well of high serum medium, and then for 24 h in low serum medium. After that, the culture medium was replaced with 1 ml/well of fresh low serum medium with or without added test substances and containing 14C-proline at 1 μCi/ml (Amersham Pharmacia Biotech), 280 μM sodium ascorbate, and 200 μM β-aminopropionitrile (both from Sigma Aldrich, St. Louis, MO). After 24 h, cell culture supernatants were collected, rapidly frozen in liquid nitrogen and freeze-dried at −70°C. The pellets were dissolved in 100 μl Laemmli buffer per 1 ml of the cell culture supernatant, and the samples were electrophoretically separated in 7.5% acrylamide gels. Fluorographic images were developed using EN' HANCE autoradiography enhancer (NEN, Boston, MA). Gel images were collected using Storm densitometer (Molecular Dynamics, Sunnyvale, CA), and band densities were analyzed with ImageQuant software (Molecular Dynamics). The identity of two major collagen bands was confirmed by sensitivity to collagenase (Sigma Aldrich) digestion.

Differences in fibroblast response to stimulation were evaluated using two-tailed t test. To confirm synergy, the observed response to the combined factors was compared with the response to be expected based on the additive action of the factors.

Immunoblotting for tyrosine phosphorylation of signal transduction factors

Fibroblasts were plated in 6-well plates (Costar) at 2 × 10^5 cells per well in 3-ml culture in high serum tissue culture medium overnight. Then, the medium was replaced with low serum medium and fibroblasts incubated for another 24 h. After incubation with IL-4 and CD40L for 1–30 min, fibroblast cultures were washed with ice-cold PBS containing 100 μM Na_3VO_4. Fibroblasts were lysed with 1 ml ice-cold lysis buffer containing 0.5% Nonidet P-40, 1 mM EDTA, 1 mM Na_3VO_4, 50 mM NaF, and 10% protease inhibitor mixture (Sigma Aldrich) in PBS for immunoprecipitation, or were lysed in 250 μl of Laemmli sample buffer for analysis of overall pattern of tyrosine phosphorylation. For immunoprecipitation, the lysates were incubated with 1 μg the desired Ab for 1 h on ice. Immune complexes were precipitated with protein A-Sepharose beads (Amersham Pharmacia Biotech) or Protein A-agarose (Sigma Aldrich) for 5 min at 4°C. Laemmli samples were denatured by heating at 95°C for 5 min. Electrophoretic separation of immunoprecipitated proteins or cell lysates was done in 7.5% acrylamide gels, and bands were transferred onto Immobilon NC membranes (Millipore, Bedford, MA). Membranes were probed with specific Abs at 1/200 dilution and visualized with an ECL detection system (Pierce, Rockford, IL) that was used according to the manufacturer’s directions. Anti-phosphotyrosine mAb (4G10) and Abs against IRS-1 and IRS-2 were purchased from Upstate Biotechnology (Lake Placid, NY). Abs against IL-4Ra, JAK1, JAK3, and gp130 were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Gel images were collected using a Storm densitometer and band densities analyzed with ImageQuant software (Molecular Dynamics). Two inhibitors of intracellular signal transduction, genistein and PD98059, were purchased from Sigma Aldrich.

IL-6 and TGF-β1 assays

ELISPOT assays for IL-6 and TGF-β1 production by fibroblasts were performed using Unifilter Polylfluorotolcan ELISPOT plates (Whatman, Clifton, NJ), according to the manufacturer’s instructions. Briefly, the wells on the ELISPOT plates were coated with capture Abs diluted in PBS and then blocked with 1% BSA in PBS. Fibroblasts were cultured at 2 × 10^5 cells/ml in 100 μl cell cultures, alone or with the desired test substances, and incubated for 2–5 days at 37°C in humidified 5% CO₂. Anti-IL-6 and anti-TGFB-1 capture and developing Abs were purchased from R&D Systems (Minneapolis, MN). Streptavidin-alkaline phosphatase conjugate (Upstate Biotechnology) and nitro blue tetrazolium substrate (Sigma Aldrich) were used for color development. The resulting spots were counted on a computer-assisted image analyzer (Autoimmun Diagnostika, Beltsville, MD). Data were expressed as spot counts ± SD of quadruplicate cultures.

Expression of cell surface molecules

Expression of IL-4Ra, CD40, and common γ-chain on fibroblasts was studied by immunofluorescence using FITC-labeled specific Abs (BD Pharmingen). Fibroblasts were adhered to chamber slides (Lab-Tek, Nalg Nunc, Naperville, IL) at 2 × 10^5 cells/ml, preincubated overnight in 10% high serum tissue culture medium, and then activated for 3 days with IL-4 and soluble CD40L, or mouse monoclonal anti-CD40, alone and in combinations. Cells were fixed with 2% paraformaldehyde in PBS, pH 7.4. Slides were then blocked with 5% goat serum and stained with specific Abs. Nuclei were stained with propidium iodide. Digital images were acquired using a Nikon Microphot FX fluorescent microscope equipped with Sony DKC CCD camera, magnification >200X using uniform setting for all slides. Images were exported into a personal computer, and the intensity of surface staining was analyzed using IPLab imaging software (Scanalytics, Fairfax, VA). Mean density of staining was calculated for 50 cells on each slide and data were compared using a t test.

Results

Synergistic stimulation of fibroblast proliferation by CD40 ligation and IL-4

Fibroblast proliferation was up-regulated by IL-4 in a dose-dependent fashion from 1 to 100 U/ml, with maximum increase in proliferation of 2.5-fold. Recombinant human soluble CD40L and anti-CD40 mAb used from 0.01 to 1 μg/ml caused a dose-dependent increase in proliferation, with maximum increase of 2-fold. rIFN-γ inhibited proliferation 1.3-fold at 500 U/ml and 1.7-fold at 1000 U/ml. These fold-changes in proliferation are consistent with reports previously published by others (7, 19, 32). Based on the dose-dependence data for the factors alone, suboptimal concentrations of the factors were chosen for the experiments with the factors combined to avoid saturation of the proliferative response by each factor alone. In combinations, IL-4 was used at 10 U/ml, anti-CD40 mAb and CD40L were used at 0.1 μg/ml, and IFN-γ was used at 500 U/ml. Incubation of fibroblasts with IL-4 combined with either anti-CD40 mAb or CD40L caused an increase in proliferation with the amplitude that was higher than a combination of independent effects of IL-4 alone and anti-CD40 mAb or CD40L alone (Fig. 1, A and B provide two examples). Five fibroblast cell lines derived from the skin of different healthy individuals were studied in five or more independent experiments with each cell line. Two lines (NDF1 and NDF2) consistently demonstrated synergistic up-regulation of proliferation in response to combined CD40 ligation and IL-4, with the effect of the combination greater than the sum of the independent effects (p < 0.01 in all cases). These two lines were used in the subsequent experiments addressing the possible mechanism of synergy. In two more lines, a tendency toward synergistic response was seen in all experiments, although the difference between the calculated additive response and observed synergistic response only reached statistical
significance ($p < 0.05$) in some experiments. In one line, the proliferative response to the combined CD40 ligation and IL-4 was not synergistic.

Although the fibroblast lines demonstrated variability in levels of CD40 and IL-4Rα cell surface expression, as revealed by immunohistochemical staining, the differences among the lines in response to the combined CD40 ligation and IL-4 did not correlate with the levels of expression of these receptors. All of the studied lines responded to CD40 ligation and IL-4 alone; although the degree of response varied among the lines, the heterogeneity in responsiveness did not correlate with level of the surface receptors (data not shown).

Incubation of fibroblasts with IFN-γ caused inhibition of proliferation, which could be neutralized by CD40 ligation (Fig. 1C). Anti-CD40 Ab and IL-4 alone at chosen concentrations partially neutralized the inhibitory action of IFN-γ, whereas a combination of these two factors taken at the same concentrations reversed the overall effect from inhibition to stimulation (Fig. 1D). Thus, CD40 ligation can shift the effects of IL-4 and IFN-γ toward the proliferative action of IL-4 and against the anti-proliferative action of IFN-γ.

Stimulation of the fibroblast lines in a similar fashion led to a dose-dependent increase in collagen production in response to IL-4, with a maximum 3-fold excess in secreted collagen. In contrast, ligation of CD40 caused little increase in collagen production (0.9- to 1.2-fold, $p > 0.05$). No synergy between the two factors on collagen production was observed in any of the five lines studied; the effect of the factors combined did not exceed the effect of IL-4 alone in more than one experiment for any line (data not shown).

Changes in receptor expression and autocrine cytokine production are unlikely mechanisms of the synergy

The interactions between CD40 ligation and IL-4 could be mediated through up-regulation in IL-4Rα and/or CD40 expression. In two experiments, each with two cell lines, a 2- to 3-fold up-regulation vs control ($p < 0.05$) of IL-4Rα was detected by immunofluorescent staining after stimulation with IL-4, whereas CD40 ligation had no effect on IL-4Rα expression (1.0- to 1.3-fold stimulation, $p > 0.05$), and the combined action of the two factors on IL-4Rα expression was not different from the effect of IL-4 alone (0.8- to 1.2-fold difference, $p > 0.05$; example shown in Fig. 2). Similarly, CD40 expression was not increased by IL-4 or CD40 ligation, alone or in combinations ($p > 0.05$ for all comparisons; data not shown), confirming earlier reports (31). This lack of synergistic increase in IL-4Rα and CD40 expression was confirmed by Western blotting of fibroblast lysates for these proteins (data not shown). Thus, up-regulation in IL-4Rα or CD40 expression...
stimulated with IL-4, IFN-\(/

Another potential mechanism of CD40 ligation-IL-4 synergy could involve an increased production of autocrine factors that stimulate fibroblast proliferation. Two cytokines have been implicated as autocrine profibrotic factors, TGF-\(\beta\) (e.g., Ref. 41) and IL-6 (7, 42, 43). Despite antagonistic action of IL-4 and IFN-\(\gamma\) on fibroblasts, ELISPOT experiments in both studied cell lines showed that both cytokines in tested concentrations up-regulated production of IL-6 by fibroblasts \(\sim 1.5\)-to 2-fold \((p < 0.05)\), as did anti-CD40 mAb (Fig. 3), which is consistent with published data for all three factors (7, 32, 44). There was no synergy of anti-CD40 mAb or CD40L with either cytokine on stimulated IL-6 production (the range of the effect was a 0.8- to 1.3-fold stimulation in comparison with either IL-4 or IFN-\(\gamma\) alone, \(p > 0.05\), data not shown). Production of another autocrine cytokine, TGF-\(\beta\), was at the low threshold of detection in control cultures, with the positive control of PBMC showing high levels of TGF-\(\beta\) producing cells. Production of TGF-\(\beta\) was not affected by either CD40 ligation or IL-4, alone or in combinations (data not shown). Thus, the observed combined effects were unlikely to be explained by an autocrine cytokine mechanism involving IL-6 or TGF-\(\beta\).

Protein tyrosine kinase signaling in fibroblasts is synergistically activated by CD40 ligation and IL-4

The CD40 ligation and IL-4 synergy could be mediated by intracellular signaling. Western blotting of electrophoretically separated crude lysate of nonactivated fibroblasts revealed a scarce pattern of constitutive protein tyrosine phosphorylation, with one major band at \(\sim 130\) kDa. The level of protein tyrosine phosphorylation in this band was synergistically up-regulated by fibroblast exposure to CD40L and IL-4 (Fig. 4A). Preincubation with CD40L for 3 days did not change the background pattern of protein tyrosine phosphorylation, but affected the pattern of phosphorylation after fibroblast exposure to combined CD40L and IL-4, with preserved synergy on the level of phosphorylation in the 130 kDa band, and additional bands at \(\sim 95, 85,\) and \(75\) kDa (Fig. 4B). Preincubation with IL-4 did not cause such a change in tyrosine phosphorylation. Although preincubation with CD40L increased the amplitude of phosphorylation in response to the combination of CD40 ligation and IL-4, it had little, if any, effect on the amplitude of the proliferative response of fibroblasts to IL-4 or CD40L, either alone or combined.

To identify specific molecules contributing to increased phosphorylation in the major 130 kDa electrophoretic band in response to the combined CD40L and IL-4, immunoprecipitation-Western blotting experiments were performed. TNFR-associated factor (TRAf)\(\text{-}2\), TRAF-3, TRAF-5, and TRAF-6, which are main signaling molecules from CD40, were not considered as potential contributors to the 130 kDa phosphorylation band because of their lower molecular mass of \(\sim 55-65\) kDa (reviewed in Ref. 45). In contrast, molecules participating in intracellular signaling from IL-4, such as IL-4R, Janus kinase (JAK1), insulin receptor substrate (IRS1), and IRS2, have molecular mass of \(\sim 130\) kDa (46). Phosphorylation of IL-4R\(\alpha\), JAK1, IRS1, and IRS2 in fibroblasts was synergistically up-regulated in response to the combined CD40L and IL-4, whereas phosphorylation of an irrelevant cytokine receptor chain, gp130, did not change (Fig. 5). JAK3 was not detected in the studied fibroblast lines (data not shown). These findings suggest that CD40 ligation and IL-4 synergy on fibroblast proliferation may be mediated through changes in IL-4 protein tyrosine kinase signaling.

To confirm that tyrosine kinase signaling was involved in this synergy, fibroblasts were stimulated with anti-CD40 mAb, IL-4, or the combination, with or without two pharmacologic inhibitors of intracellular signaling. Genistein, an inhibitor of protein tyrosine kinases, and PD98059, an inhibitor of mitogen-activated protein (MAP) kinase kinase, were both used at 30 \(\mu\)M concentration. Genistein caused 65–70% inhibition of fibroblast proliferation, whereas PD98059 was less effective, causing only 25–30% inhibition (data not shown). Thus, it appears that tyrosine phosphorylation was involved in signaling from CD40 and IL-4.

Discussion

Synergy of CD40 ligation and IL-4 in the regulation of B cell proliferation and isotype switching is well established (36–38). Similarly, these two factors synergize in up-regulation of adhesion molecule expression on endothelial cells (39). This work demonstrates that such synergy extends to fibroblasts (Fig. 1). Synergistic up-regulation of fibroblast proliferation by CD40 ligation and IL-4 could be mediated by T cells, mast cells, eosinophils, and basophils, all of which can express CD40L and secrete IL-4. Such synergy can potentially contribute to regulation of fibroblast activities in health and pathology, such as normal wound healing, the formation of hypertrophic scars (2), and the development of tissue fibrosis following inflammation (25).

The stimulating effect of IL-4 on fibroblast proliferation can be opposed by an inhibitory action of IFN-\(\gamma\) (Fig. 1). In vivo, both

![Figure 3](http://www.jimmunol.org/)

**FIGURE 3.** IL-6 production by NDF1 fibroblast cultures. Cells were stimulated with IL-4, IFN-\(\gamma\), anti-CD40 mAb, and IL-4 plus anti-CD40 mAb combination for 2 days. Data are shown as quadruplicate mean counts of IL-6 producing cells \(\pm SD\), as detected by ELISPOT assay.

![Figure 4](http://www.jimmunol.org/)

**FIGURE 4.** Western blotting analysis of phosphorylation patterns in NDF1 fibroblasts. Electrophoretically separated fibroblast crude lysates were stained using anti-phosphotyrosine Abs (A and B) and anti-gp130 Ab (C). A. From left to right, fibroblasts were activated for 12 min with culture medium, IL-4, CD40L, and IL-4 and CD40L combined. B. Fibroblasts were pre-stimulated for 72 h with CD40L, 0.1 \(\mu\)g/ml, and then activated as in A. C. Equal sample loading for A was confirmed by staining with anti-gp130 Ab. Equal loading in B was confirmed in the same manner (data not shown). Neither IL-4 nor CD40 ligation have been reported to affect the expression of gp130 protein in fibroblasts.
IL-4 and IFN-γ may coexist in healthy tissues and at sites of inflammation. A shift in the ratio of IL-4 and IFN-γ toward more IL-4 can be associated with increased fibrosis (e.g., in Ref. 25). It appears, based on the experiments reported in this study, that CD40 ligation on fibroblasts may shift the functional balance between IL-4 and IFN-γ toward the effects of IL-4, thus promoting fibrosis without changing the actual ratio of IL-4 to IFN-γ.

In B cells, synergy between CD40 ligation and IL-4 is mediated through up-regulation of expression of surface molecules, such as IL-4Rα, and changes in signaling, particularly the expression and phosphorylation of JAK3 (47, 48). This report shows that changes in expression of IL-4Rα or CD40 do not mediate the synergy between CD40 ligation and IL-4 on fibroblast proliferation. Indeed, this work confirms the previously reported observation that exposure to IL-4 does not increase the level of CD40 expression on fibroblasts (31). In addition, this work shows that although exposure to IL-4 increases expression of IL-4Rα on fibroblasts, CD40 ligation does not exert such an effect, nor does CD40 ligation synergize with IL-4 in up-regulating IL-4Rα expression (Fig. 2).

To address the possibility that changes in signaling contribute to the synergy between CD40 ligation and IL-4, tyrosine phosphorylation of signaling molecules was tested in fibroblast lysates (Fig. 4). These experiments revealed that CD40 ligation and IL-4 synergistically increase phosphorylation of several proteins, with a particularly strong band at 130 kDa. To identify proteins that might contribute to that band, molecules known to be involved in IL-4R or CD40 signaling were considered. Many of IL-4R signaling pathway molecules have a molecular mass close to 130 kDa, including JAK1, IRS1, IRS2, and IL-4Rα itself (49–51). In contrast, CD40 signaling molecules, TRAFs, have lower molecular mass of 55–65 kDa (reviewed in Ref. 45). Of note, IL-4R signaling through IRS1 and IRS2 molecules activates proliferation (50, 51), whereas signaling through STAT6 (calculated molecular mass, 93 kDa), leads to an increase in gene translation, but not proliferation. Immunoprecipitation of fibroblast lysates with Abs against IL-4Rα, JAK1, IRS1, and IRS2 and subsequent Western blotting for tyrosine phosphorylation demonstrated that phosphorylation of each of these factors is synergistically up-regulated by CD40 ligation and IL-4 (Fig. 5). Thus, CD40 ligation increases signaling through the IL-4R pathway in fibroblasts, leading to synergistic effect of CD40 ligation and IL-4 on fibroblast proliferation.

Some aspects of IL-4R signaling in fibroblasts appear to be different from that in hematopoetic cells, including B cells. Particularly interesting is the observation in this report of a synergistic increase in IL-4Rα phosphorylation after stimulation of fibroblasts with combined CD40L and IL-4 (Fig. 5). In contrast to B cells (47), this effect is not caused by stimulation of IL-4Rα expression by CD40 ligation (Fig. 2). It is possible that ligation of CD40 increases IL-4R sensitivity without increasing its level of expression, similar to the effects of CD28 ligation on Th2 cells (52). Also indicative of differences in IL-4 signaling between fibroblasts and hematopoetic cells is that JAK3 appears to be involved in CD40 ligation-IL-4 synergy in B cells, with an increase in its expression and a change in phosphorylation/dephosphorylation kinetics induced by CD40 ligation (47, 48). However, this work found that neither JAK3 nor common γ-chain (our unpublished data), with which JAK3 is associated in IL-4R signaling, are expressed in the dermal fibroblasts. Additional differences in IL-4R signaling between fibroblasts and hematopoetic cells include involvement of STAT1 and STAT3 in fibroblast signaling (53), two factors conventionally associated, respectively, with IFN-γ and IL-6/IL-10. Unlike its effects in hematopoetic cells, IL-4 increases phosphorylation and activity of JNK and extracellular signal-regulated kinases in fibroblasts (54). Finally, IL-4 stimulation may be inhibited by a naturally occurring splice variant, IL-4Rβ (55), in T cells (56), B cells, and monocytes (57), whereas IL-4 and IL-4Rβ both stimulate fibroblasts to increase collagen mRNA production (25). Further investigation is necessary to address the effects of CD40 ligation on the consequences of fibroblast exposure to IL-4 and the mechanisms by which these effects occur.

The contribution of autocrine regulation of fibroblast proliferation was tested as another possible mechanism of synergy between CD40 ligation and IL-4. Autocrine TGF-β is believed to be an important regulator of fibroblast proliferation (41, 58). There was very low production of TGF-β1 in these fibroblast lines, and it was not stimulated by CD40 ligation, IL-4, or combinations thereof. Another potential autocrine fibroblast regulator is IL-6. IL-4 stimulates production of IL-6 by fibroblasts (7), and IL-6 may stimulate fibroblast proliferation (42, 43). In B cells and endothelial cells, CD40 ligation and IL-4 synergistically increase production of IL-6 (37, 39). However, autocrine IL-6 production does not appear to be involved in this synergistic response, because both IL-4 and IFN-γ increase production of IL-6 by fibroblasts (Fig. 3), confirming previous reports (7, 44), although they have opposite effects on fibroblast proliferation (Fig. 1). There was no synergy detected by ELISPOT between CD40L and IL-4 in up-regulating IL-6 production in NDF1 and NDF2 lines under the studied conditions.


References


