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Tumor Necrosis Factor (TNF) α Increases Collagen Accumulation and Proliferation in Intestinal Myofibroblasts via TNF Receptor 2*

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Intestinal fibrosis is an incurable complication of Crohn's disease involving increased numbers of collagen-producing myofibroblasts. Tumor necrosis factor (TNF) α has defined proinflammatory roles in Crohn's disease but its role in fibrosis is unclear. We tested the hypothesis that TNFα increases collagen accumulation and proliferation in intestinal myofibroblasts and has additive effects in combination with insulin-like growth factor (IGF) I. The mechanisms, TNF receptor isoform, and downstream signaling pathways were examined. Intestinal myofibroblasts from wild-type (WT) mice or mice homozygous for disruption of genes encoding TNFR1 (TNFR1$^{-/-}$), TNFR2 (TNFR2−/−), or both (TNFR1/2$^{-/-}$), were treated with TNFα, IGF-I, or both. In WT cells, TNFα and IGF-I stimulated type I collagen accumulation and DNA synthesis in an additive manner. IGF-I, but not TNFα, stimulated type I collagen gene activation. TNFα, but not IGF-I, induced tissue inhibitor of metalloproteinase-1 (TIMP-1) expression and reduced matrix metalloproteinases-2 activity and collagen degradation. TNFα also activated ERK1/2. These responses to TNFα were absent in TNFR2$^{-/-}$ and TNFR1/2$^{-/-}$ myofibroblasts, whereas TNFR1$^{-/-}$ cells showed similar responses to WT. Inhibition of ERK1/2 diminished TNFα-induced DNA synthesis in WT and TNFR1$^{-/-}$ cells. Differences in TNFα-induced STAT3/DNA binding activity and not NFκB and AP-1 transcriptional activation correlated with impaired collagen accumulation/TIMP-1 induction in TNFR2$^{-/-}$ cells. Constitutively active STAT3 rescued TIMP-1 expression in TNFR2$^{-/-}$ cells. We conclude that TNFα and IGF-I may additively contribute to fibrosis during intestinal inflammation. TNFR2 is a primary mediator of fibrogenic actions of TNFα acting through ERK1/2 to stimulate proliferation and through STAT3 to stimulate TIMP-1 and inhibit collagen degradation.

Fibrosis is a major and serious complication of Crohn's disease, an incurable, chronic inflammatory disease of the gastrointestinal tract. Fibrosis results from an imbalance between synthesis and degradation of collagen and other extracellular matrix components, ultimately leading to increased net matrix accumulation within the bowel wall. Fibrosis is thought to be an overactive, irreversible wound-healing response to chronic inflammation (1) and can lead to the serious complications of stricture, bowel narrowing and/or obstruction. In the normal intestine, subepithelial myofibroblasts, and fibroblasts in the submucosa, intermuscle connective tissue of the muscularis propria, and serosa are the primary sites of collagen mRNA and protein expression (2, 3). In intestine of patients with Crohn's disease, myofibroblasts are increased in number in all bowel layers and colocalize with sites of type I collagen mRNA expression and collagen deposition (2). These results suggest that myofibroblasts are cellular mediators of intestinal fibrosis in Crohn's disease. Wound healing and fibrosis in other organs also involves myofibroblasts (4–6).

Tumor necrosis factor α (TNFα)[2] is a central mediator of chronic inflammatory diseases including Crohn's disease, as evidenced from clinical studies reporting a dramatic improvement in Crohn's patients treated with infliximab (Remicade; Centocore, Inc.), a neutralizing monoclonal antibody against TNFα (7, 8). The role of TNFα in inflammation-induced fibrosis appears complex because available evidence suggests that TNFα regulates collagen synthesis in a tissue-specific manner. In hepatic stellate cells during liver injury, several studies indicate anti-fibrogenic actions of TNFα via inhibition of collagen α1(I) gene expression (9–12). However, increasing evidence indicates TNFα as a mediator of inflammation-induced fibrosis in other systems. Mice deficient in either the 55- (TNFR1) or 75-kDa (TNFR2) TNF receptor show reduced experimentally induced pulmonary fibrosis (13) or renal fibrosis (14). The reduced pulmonary fibrosis in TNF receptor null mice occurred despite similar increases in type I collagen gene expression as wild-type mice, but was accompanied by attenuated induction of tissue inhibitor of metalloproteinase-1 (TIMP-1) mRNA (13). TIMP-1 is an inhibitor of matrix metalloproteinases (MMPs) that cleave various components of the extracellular matrix and therefore reduces collagen degradation. These findings in TNFR null mice suggest that TNFα normally plays a role in up-regulation of TIMP-1 during lung injury and may promote fibrosis by inhibiting collagen degradation. However, one complication in these *in vivo* studies is that direct effects of TNF receptor knock-out on fibrosis could not be separated from effects on inflammation and the receptor mediating TNFα action on fibrosis was not defined.

Little is known about the role of TNFα in inflammation-induced intestinal fibrosis. Given the key role of TNFα as a mediator of Crohn's disease as evidenced by the therapeutic benefits of TNFα inhibitors, and the fact that fibrosis is a common and serious problem in Crohn's disease, defining the role of TNFα in inflammation-induced intestinal fibrosis is of considerable clinical significance. Because it is difficult to

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[2] The abbreviations used are: TNFα, tumor necrosis factor α; TNFR, tumor necrosis factor receptor; TIMP-1, tissue inhibitor of metalloproteinases; MMP, matrix metalloproteinase; IGF-I, insulin-like growth factor; STAT, signal transducers and activators of transcription; ERK, extracellular signal-regulated kinase; WT, wild-type; GFP, green fluorescent protein; PBS, phosphate-buffered saline; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

distinguish direct effects of TNFα on intestinal fibrosis *in vivo* from those secondary to effects on inflammation, this study utilized cultured intestinal myofibroblasts, cells believed to be major cellular mediators of fibrosis, to determine TNFα action and its mechanism of action on collagen accumulation and proliferation.

Although TNFR1 is regarded as the primary signaling receptor for systemic TNFα inflammatory responses (15, 16), TNFR2 may mediate TNFα effects in a paracrine or autocrine manner because it is strongly activated by membrane-bound TNFα (17). Evidence for distinct roles of TNFR1 and TNFR2 in intestinal inflammation has recently started to emerge. In patients with Crohn's disease, lamina propria T cells show increased levels of TNFR2 and not TNFR1 (18). TNFR2 expression is increased in colonic epithelial cells in human inflammatory bowel disease and in mouse models of colitis (19). SCID mice reconstituted with T cells overexpressing TNFR2 show enhanced and accelerated development of colitis (18). Although these data indicate a role of TNFR2 in intestinal inflammation, the roles of TNFR1 and TNFR2 in intestinal fibrosis are not well defined. This study utilized cultured intestinal myofibroblasts isolated from TNFR1$^{-/-}$, TNFR2$^{-/-}$, and TNFR1/2$^{-/-}$ mice to determine which TNF receptor mediates pro-fibrogenic actions of TNFα.

Considerable evidence points to a role of IGF-I as a mediator of fibrosis in the intestine. Increased local expression of IGF-I mRNA occurs in all layers of inflamed or fibrotic intestine of Crohn's disease patients but not in healthy portions of intestine from the same patients (2, 20, 21). Local expression of IGF-I mRNA is increased in multiple animal models of intestinal inflammation (22–25). Increased IGF-I expression is localized to myofibroblasts and smooth muscle cells at sites of increased collagen mRNA expression and fibrosis in intestine of Crohn's disease patients and animal models of chronic enterocolitis (2, 22, 23, 26). IGF-I stimulates collagen protein synthesis and proliferation of enteric smooth muscle cells (26) and intestinal myofibroblasts *in vitro* and *in vivo* (27–29). Together, these data indicate that locally expressed, mesenchymal cell-derived IGF-I contributes to inflammation-induced intestinal fibrosis. However, the possibility that IGF-I interacts with TNFα to regulate collagen synthesis or proliferation in intestinal myofibroblasts has not been previously tested. This study utilized intestinal myofibroblasts isolated as a simple system to define whether TNFα affects collagen synthesis and proliferation and if there are interactions between TNFα and IGF-I. We provide novel evidence that TNFα increases collagen accumulation and has mitogenic effects in intestinal myofibroblasts and that these effects are mediated via the TNFR2. We also show that TNFα has additive effects with IGF-I on collagen accumulation and proliferation and that TNFR2 is essential for these responses. Finally, we demonstrate that the lack of mitogenic and fibrogenic actions in TNFR2$^{-/-}$ cells are associated with altered ERK1/2 and STAT3 activation, respectively.

EXPERIMENTAL PROCEDURES

Materials—Mouse recombinant TNFα was purchased from Peprotech (Rocky Hill, NJ) and recombinant human IGF-I from Genentech (San Francisco, CA). Rabbit polyclonal antibody specific for procollagen α1(I) was purchased from Rockland Immunochemicals (Gilbertsville, PA); rabbit polyclonal antibody to actin from Sigma; peroxidase-conjugated donkey anti-rabbit from Jackson ImmunoResearch Laboratories (West Grove, PA); phospho-ERK1/2 and total ERK1/2 from Santa Cruz (Santa Cruz, CA); enhanced chemiluminescence (ECL) reagent from PerkinElmer Life Sciences; goat anti-rabbit secondary antibody conjugated to an infrared dye (IR Dye 800 Conjugated Anti-rabbit IgG) from Rockland; [^{3}H]thymidine and [^{32}P]UTP from Amersham Biosciences; and STAT3 consensus gel shift oligonucleotides from Santa Cruz.

Mice—Wild-type (WT), TNFR1$^{-/-}$ (TNFR1a), and TNFR2$^{-/-}$ (TNFR1b) mice on the inbred C57BL/6 background were purchased from The Jackson Laboratory (Bar Harbor, ME). WT and mice deficient in both TNFR1 and TNFR2 signaling (TNFR1/2$^{-/-}$; TNFR1a/b) on the mixed C57BL/6/129S background were also purchased from The Jackson Laboratory. No differences were noted between wild-type cells of the pure C57BL/6 background and the mixed C57BL/6/129S backgrounds in any of the experiments, and therefore data from both wild-type strains were pooled. Mice on the inbred C57BL/6 background expressing a transgene comprising the basal promoter of the murine procollagen α1(I) collagen gene (−3122 to +111 relative to the transcription start site) and two upstream DNase-sensitive sites that appear to be important for maximal cell-specific expression linked to the GFP transgene were derived as previously described (30). Separate cell lines were isolated from three mice of each genotype for use in subsequent studies.

Isolation of Intestinal Myofibroblasts—Intestinal myofibroblasts from adult (40–50-day-old) WT, TNFR1$^{-/-}$, TNFR2$^{-/-}$, TNFR1/2$^{-/-}$, and collagen-GFP transgenic mice were isolated and cultured as described previously (31). Myofibroblast phenotype was confirmed based on positive expression for vimentin and α-smooth muscle actin as evaluated by Western immunoblot (32, 33). Subconfluent cells were studied at passages 3–8. Verification of TNF receptor knock-out was determined by Western immunoblot analysis using polyclonal anti-rabbit TNFR1 or TNFR2 antibodies from Santa Cruz Biotechnology (Santa Cruz, CA) and compared with WT cells that express both TNF receptors (data not shown).

Assays of Cell Proliferation—Incorporation of [^{3}H]thymidine into DNA was used as a measure of cell proliferation by IGF-I (10 ng/ml) and TNFα (5 ng/ml) alone or in combination. Serum-deprived cells were incubated with [^{3}H]thymidine (2 μCi/ml) and peptide for 18 h. After incubation, medium was aspirated, cells were washed with 1× PBS and fixed with 10% trichloroacetic acid. Total cell extracts were collected in 0.2 N NaOH and 0.1% SDS. Radioactivity incorporated into DNA was quantified by scintillation counting. All assays were performed in triplicate and repeated in at least three separate experiments.

Western Immunoblot for Procollagen α1(I)—Serum-deprived intestinal myofibroblasts were treated with 5 ng/ml TNFα or 10 ng/ml IGF-I alone or in combination for 48 h. Doses were determined as maximal in separate dose-response experiments (data not shown). Cells were solubilized in Laemmli's 2× SDS sample buffer and whole cell lysates were analyzed for procollagen α1(I) protein abundance and actin (loading control) as previously described (32).

In-cell Western for Collagen for Procollagen α1(I)—An in-cell Western method was also utilized in these studies to monitor for procollagen α1(I) accumulation in a subset of experiments. This method provides a highly sensitive and rapid system to assay collagen accumulation and was validated as providing comparable data to Western immunoblots in WT cells. Intestinal myofibroblasts isolated from ileum of WT, TNFR1$^{-/-}$, TNFR2$^{-/-}$, and TNFR1/2$^{-/-}$ mice were plated in an equal number in a 24-well culture plate. Serum-deprived cells were treated with IGF-I (10 ng/ml) and TNFα (5 ng/ml) alone or in combination for 48 h. Culture medium was removed by aspiration and cells were fixed with 4% formaldehyde in PBS for 20 min at room temperature. Cells were washed with 1× PBS containing 0.1% Triton X-100 and blocked in 0.1% casein and 0.2× PBS solution. Cells were incubated with a procollagen α1(I) primary antibody, washed with 1× PBS containing 0.1% Tween 20, and incubated with a secondary antibody conjugated to an

infrared dye (IR dye 800 conjugated anti-rabbit IgG). Plates were scanned to visualize signal using the Odyssey Infrared Imaging System (Li-Cor Biosciences, Lincoln, NE) in the 800-nm fluorescent channel. Signal was quantified using Odyssey Infrared Imaging System Application Software version 1.2 (Li-Cor Biosciences). Plates were reprobed for β-tubulin protein and signal was visualized in the 680-nm fluorescent channel to verify equal cell number across wells.

Collagen Gene Transcription Monitored by Activation of a Procollagen α1(I) Promoter-GFP Reporter Gene—Myofibroblasts isolated from mice expressing the collagen-GFP transgene were treated with increasing doses of IGF-I and TNFα alone or in combination for 24 h. Whole cell lysates were collected in GFP protein buffer (50 mM NaH_2PO_4, 10 mM Tris-HCl, 200 mM NaCl, pH 8.0). 500 μg of protein was assayed for GFP fluorescence using the SPEX Fluorolog-3 Research T-format Spectrofluorometer at excitation of 488 nm and emission of 510 nm. GFP fluorescent units were normalized to total protein assayed as a measure of procollagen α1(I) gene activation.

Total RNA Extraction and Northern Blot Hybridization Assays—Total RNA was extracted using TRIzol reagent (Invitrogen). Northern blot hybridization was performed as previously described (34). A TIMP-1 probe was generated by PCR using primers (sense: 5′-CCACGAATCAACGAGACC-3′ and antisense: 5′-CCAAGTGCACAAGCCTAG-3′) with the addition of a T7 RNA polymerase site 5′ to the antisense primer. The PCR product was used as a complementary DNA template to generate a [^{32}P]UTP-labeled probe using T7 RNA polymerase. To account for minor variations in RNA loading across samples, blots were reprobed for human glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA using an antisense RNA probe prepared from a commercially available DNA template (Ambion Inc., Austin, TX). Blots were scanned on a PhosphorImager (Amersham Biosciences Storm 840) and abundance of specific RNAs was quantified using ImageQuant software for Macintosh. The abundance of TIMP-1 mRNA was normalized to the abundance of GAPDH mRNA in each sample.

Assays of Collagen Synthesis and Degradation—Collagen synthesis and degradation using radioactive proline was performed as previously described with some modifications (35). Intestinal myofibroblasts were incubated for 15 min at 37 °C with serum-free medium supplemented with 0.1 mM β-aminopripionitrile and fresh 10 mM ascorbate to stimulate collagen expression. Cells were then pulsed with 100 μCi/ml [5-^{3}H]proline for 24 h during which [5-^{3}H]proline was incorporated into synthesized protein. The media containing the [5-^{3}H]proline was removed, cells were rinsed with 1× PBS and incubated with serum-free media containing cold 10 mM proline for 24 h during which [5-^{3}H]proline incorporated into protein was released as these proteins are degraded. Cells were treated with TNFα at the start of the pulse and the start of the chase and compared with no treatment control cells to determine TNFα effects on collagen synthesis and degradation. Collections were obtained at 3, 8, and 24 after the start of the pulse and 1, 3, and 24 h after the start of the chase. After the desired incubation period, the media was collected and cells were lysed in 0.11 M NaCl and 0.05 M Tris-HCl (pH 7.4). Lysates were centrifuged at 1,000 × *g* for 5 min at 4 °C, and the supernatant was added to the media collected. Cell pellets were resuspended with 0.4 M NaCl and 0.1 M Hepes (pH 7.2). Proteins in the cell lysates and media collections were precipitated with trichloroacetic acid and centrifuged 1,000 × *g* for 5 min at 4 °C. The protein pellet was dissolved in 0.2 M NaOH and aliquotted into two tubes. One tube was treated with 15 μg/ml purified *Clostridium histolyticum* collagenase (Advance Biofactures, Lynbrook, NY) at 37 °C for 2 h, whereas the other tube was untreated. The collagenase specifically degrades collagen protein, thereby releasing [^{3}H]proline incorporated into collagen protein only. Proteins were precipitated with trichloroacetic acid and centrifuged. Radioactivity in the supernatant fraction was measured by scintillation counting. To determine radioactivity incorporated in collagen, the radioactivity in the untreated aliquot was subtracted from radioactivity in the aliquot treated with the collagenase. Assays were performed in duplicate.

Gelatin Zymography—A gelatinase zymography that quantitates activity of MMP-2 and MMP-9 was used as an indirect measure of TIMP-1 activity because TIMP-1 is known to inhibit these enzymes in other systems. Conditioned media of myofibroblasts treated with or without TNFα for 16 h was collected, concentrated 4 times, and mixed with the same volume of Laemmli's 2× SDS sample buffer without reducing agent. Samples were incubated for 20 min at 37 °C and subject to SDS-PAGE on 8.5% polyacrylamide gels containing 0.1% gelatin. Gels were soaked in 50 mM Tris-HCl (pH 7.5) containing 2.5% Triton X-100 for 30 min, incubated in buffer containing 50 mM Tris-HCl, 150 mM NaCl, 10 mM $CaCl_2$, and 0.5 mM $ZnCl_2$ (pH 7.5) for 16 h at 37 °C, and stained with 0.1% Coomassie Blue R-250. Intensity of gelantinolytic bands was quantified using NIH Image software (version 1.61).

ERK1/2 Activation and Role in TNFα-induced Intestinal Myofibroblast Proliferation—Western immunoblot assays were used to monitor activation of ERK1/2. Serum-deprived cells were treated with 5 ng/ml TNFα for 30, 60, and 180 min. Because maximal activation of ERK1/2 occurred after 30 min of treatment, subsequent experiments were performed at this time point. EGF treatment (10 ng/ml) was used as a positive control. An ERK1/2-specific inhibitor, PD98059 (Calbiochem, La Jolla, CA), was used to determine whether TNFα-induced [^{3}H]thymidine incorporation into DNA of WT and TNFR1$^{-/-}$ myofibroblasts was dependent on ERK1/2 signaling. Serum-deprived cells were treated with various concentrations of PD98059 for 30 min and subsequently treated with TNFα (5 ng/ml) for 18 h. Incorporation of [^{3}H]thymidine into DNA was assayed as described above.

NFκB and AP-1 Activation—Adenovirus based NFκB-luciferase and AP-1-luciferase reporter constructs were used to monitor NFκB (Ad.NFκBLuc) and AP-1 (Ad.AP-1Luc) activation, two major pathways linked to TNFα action and potentially to fibrogenic actions of TNFα. Both Ad.NFκBLuc (constructed by John F. Engelhardt, The University of Iowa, Iowa City, IA) and Ad.AP-1Luc (constructed by Drs. Paul B. McCray and Hong Peng Jia, The University of Iowa, Iowa City, IA) were generous gifts of Dr. Scott H. Randell (University of North Carolina, Chapel Hill, NC). The Ad.NFκBLuc adenoviral construct contains the firefly luciferase gene driven by four tandem copies of the NFκB consensus sequence linked to a TATA-like promoter from the herpes simplex virus-thymidine kinase gene (36, 37). The Ad.AP-1Luc was constructed as previously described (38, 39) and contains the firefly luciferase gene driven by six tandem copies of the AP-1 enhancer sequence fused to a TATA box promoter from the herpes simplex virus-thymidine kinase gene. Intestinal myofibroblasts were infected with recombinant adenoviral constructs at a multiplicity of infection ranging from 1,000 to 1,500 particles/cell in serum-free media (Dulbecco's modified Eagle's medium supplemented with 100 units/ml penicillin and 5 μg/ml transferrin). Virus was removed 24 h post-infection and cells were treated with various concentrations of TNFα and IGF-I alone or in combination for 8 h. Cells were harvested in Cell Culture Lysis Reagent (Promega) and samples were stored at −20 °C until assayed. 10 μg of total protein from each sample was measured for luciferase expression using the LUMIstar Galaxy microplate luminometer (BMG Labtechnologies, Durham, NC) with a dual injector system, in which one injector delivers assay buffer (25 mM glycylglycine, 15 mM KPO_4, 15 mM $MgSO_4$, 4 mM EGTA, 2 mM ATP, and 1 mM dithiothreitol added before

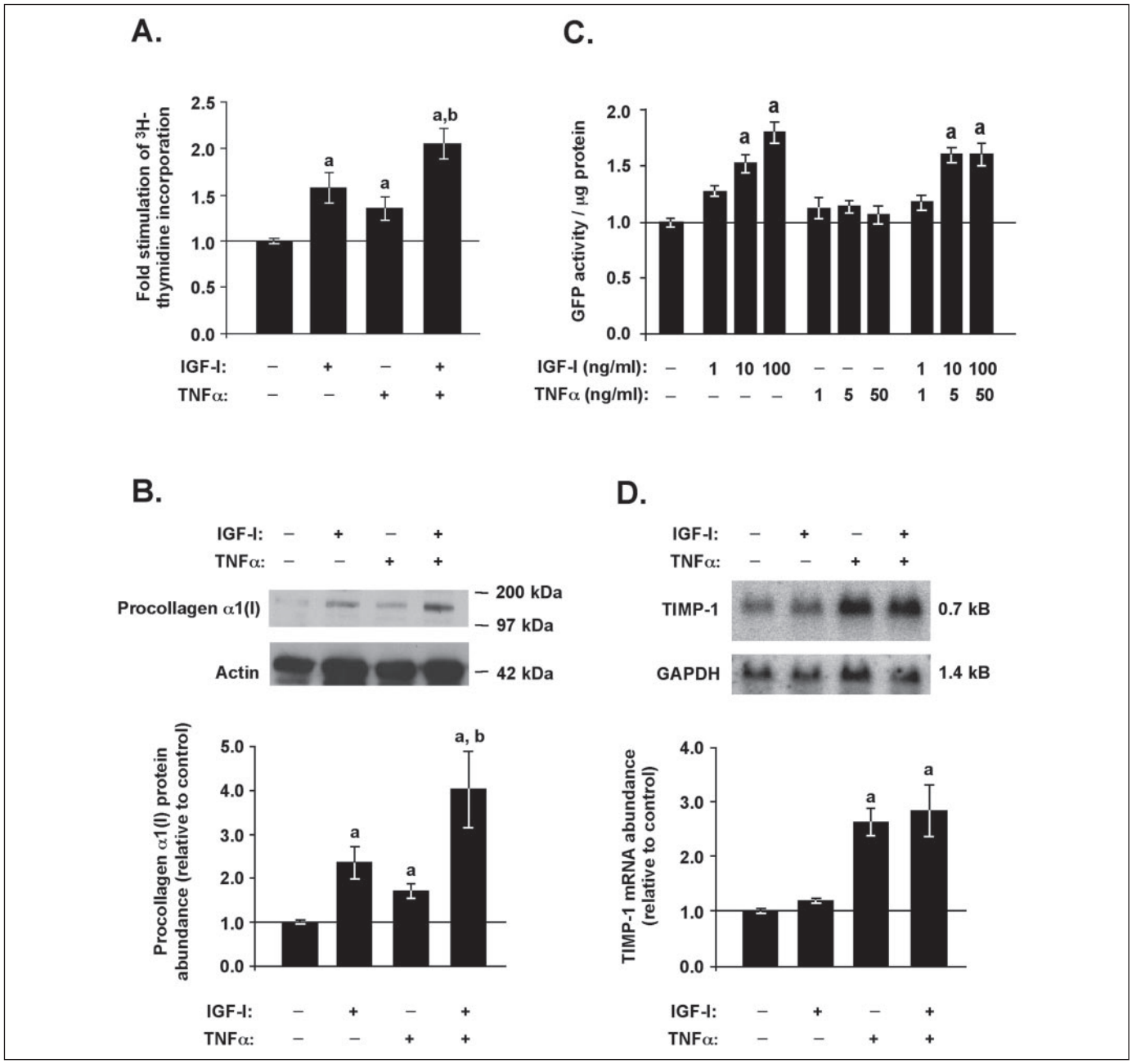

FIGURE 1. **TNFα increases DNA synthesis, collagen accumulation, and TIMP-1 expression in intestinal myofibroblasts and acts additively with IGF-I.** *A*, -fold stimulation of [^{3}H]thymidine incorporation by IGF-I (10 ng/ml) and TNFα (5 ng/ml) alone or in combination relative to no treatment controls measured in the same assay. *B*, representative Western immunoblots for procollagen α1(I) and actin (control) protein in cells treated with IGF-I (10 ng/ml), TNFα (5 ng/ml), or IGF-I + TNFα. Histograms show mean ± S.E. *C*, increasing concentrations of IGF-I or TNFα alone or in combination were used to determine whether activation of the procollagen α1(I) promoter showed a dose-dependent response to either factor. Histograms showing mean ± S.E. of the -fold stimulation of GFP fluorescence per μg of protein relative to no treatment controls. *D*, representative Northern blots showing TIMP-1 and GAPDH (control) mRNA abundance in total RNA isolated from cells treated with IGF-I (10 ng/ml), TNFα (5 ng/ml), or IGF-I + TNFα. Histograms show mean ± S.E. *a*, $p < 0.05$ *versus* no treatment; *b*, $p < 0.05$ *versus* IGF-I or TNFα alone. $n = 5$ per treatment group.

use) and the other injector delivers 1 mM D-luciferin (Sigma). All assays were performed in triplicate.

STAT3 Activation and Role in TNFα-induced TIMP-1 Expression—Electromobility shift assays were used to monitor STAT3 activation by TNFα as previously described (40). Nuclear protein extracts were isolated from serum-deprived intestinal myofibroblasts treated with 5 ng/ml TNFα for 60 min. 20 μg of nuclear protein were assayed for DNA binding to ^{32}P-labeled, double-stranded oligonucleotides corresponding to a consensus STAT3 response element binding site (Santa Cruz). Oligonucleotides corresponding to a nonconsensus STAT3 response element binding site (5′-TGCTTCCTGGACAGATC-3′) located in the TIMP-1 promoter (41) were obtained from Integrated DNA Technologies (Coralville, IA).

Serum-deprived cells were infected with constitutively active STAT3 adenovirus (Ad5STAT3C), which contains mutations C661A and C663N allowing activation of STAT3 without phosphorylation at Tyr^{705} (42), at an multiplicity of infection of 50, which was optimal for maximal infection as determined by Western immunoblot for STAT3 protein expression. Virus was removed 24 h post-infection and cells were treated with 5 ng/ml TNFα for 16 h. Total RNA was extracted using TRIzol reagent and Northern blot hybridization for TIMP-1 expression was performed as described above.

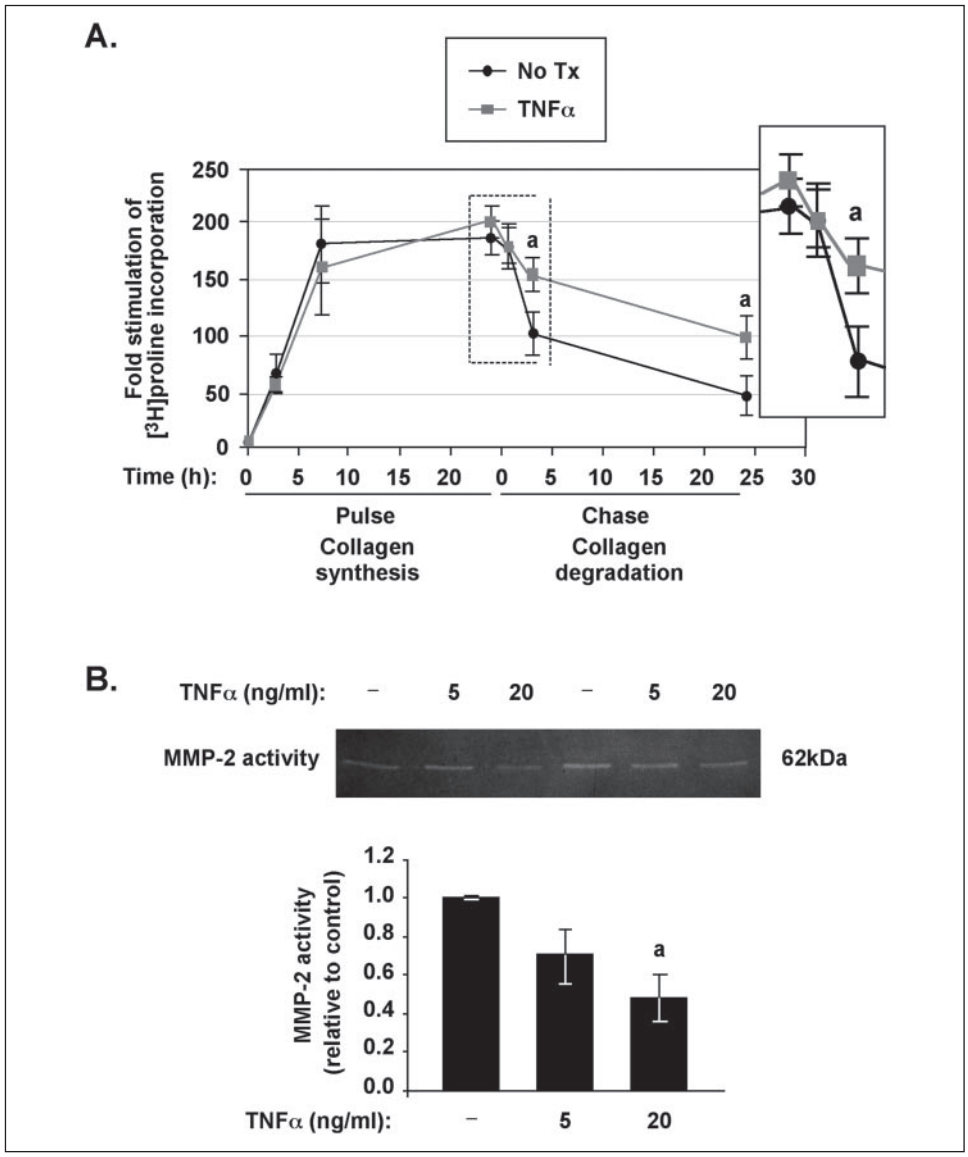

FIGURE 2. **TNFα decreases collagen degradation and MMP-2 activity in intestinal myofibroblasts.** *A*, effect of TNFα treatment (5 ng/ml) compared with no treatment (*No Tx*) on incorporation of [^{3}H]proline into collagen protein (*pulse*) as a measure of collagen synthesis and following addition of non-radioactive proline (*chase*) as a measure of collagen degradation. *Dashed box* indicates the area of enlarged inset showing decreased collagen degradation with TNFα treatment between the 1- and 3-h chase time points. *a*, $p < 0.05$ *versus* No Tx; $n = 4$ per treatment group. *B*, gelatin zymography showing effect of TNFα treatment on MMP-2 activity. *a*, $p < 0.05$ *versus* No Tx; $n = 3$ per treatment group.

Statistical Analyses—Values are expressed as mean ± S.E. Comparisons between treatments in WT cells were analyzed by one-way analysis of variance for significant effects of treatments *versus* control, followed by planned pairwise comparisons. Comparisons between treatments and TNFR genotype were analyzed by two-way analysis of variance for significant effects of treatment or genotype, and a significant interaction between treatment and genotype, which provides statistical evidence for an effect of genotype on the response to TNFα. Subsequent pairwise comparisons used Tukey's post-hoc test to test for significant differences between two particular groups. A p value < 0.05 was considered statistically significant in all analyses.

RESULTS

TNFα Increases DNA Synthesis, Collagen Accumulation, and TIMP-1 Expression in Intestinal Myofibroblasts and Acts Additively with IGF-I—We have previously shown that IGF-I induces cell proliferation and procollagen α1(I) protein accumulation in cultured intestinal myofibroblasts (32), however, the effects of the proinflammatory cytokine TNFα were unknown. [^{3}H]Thymidine incorporation into DNA was assayed to determine whether TNFα had mitogenic effects on intestinal myofibroblasts. TNFα alone increased DNA synthesis compared with no treatment controls (Fig. 1*A*). IGF-I and TNFα had an additive effect on DNA synthesis when given in combination (Fig. 1*A*). Both IGF-I and TNFα treatment induced collagen protein accumulation in intestinal myofibroblasts compared with no treatment controls (Fig. 1*B*). IGF-I and TNFα had an additive effect on collagen accumulation when given in combination (Fig. 1*B*).

Intestinal myofibroblasts isolated from ileum of mice expressing the GFP transgene linked to the procollagen α1(I) promoter were used to determine whether the increased collagen protein accumulation by IGF-I and TNFα was because of changes in gene transcription. GFP reporter expression was assayed as a measure of procollagen promoter activation in total protein extracted from cells treated with increasing doses of IGF-I or TNFα alone or in combination. IGF-I stimulated procollagen promoter activation in a dose-dependent manner, whereas TNFα had no significant effect (Fig. 1*C*). Procollagen promoter activation in cells treated with TNFα and IGF-I in combination was similar to levels observed with IGF-I alone (Fig. 1*C*). These data indicate that the effects of IGF-I, but not TNFα, on collagen accumulation involve transcriptional activation of the procollagen α1(I) gene.

To determine whether the increased collagen protein accumulation in response to TNFα and/or IGF-I was because of post-transcriptional

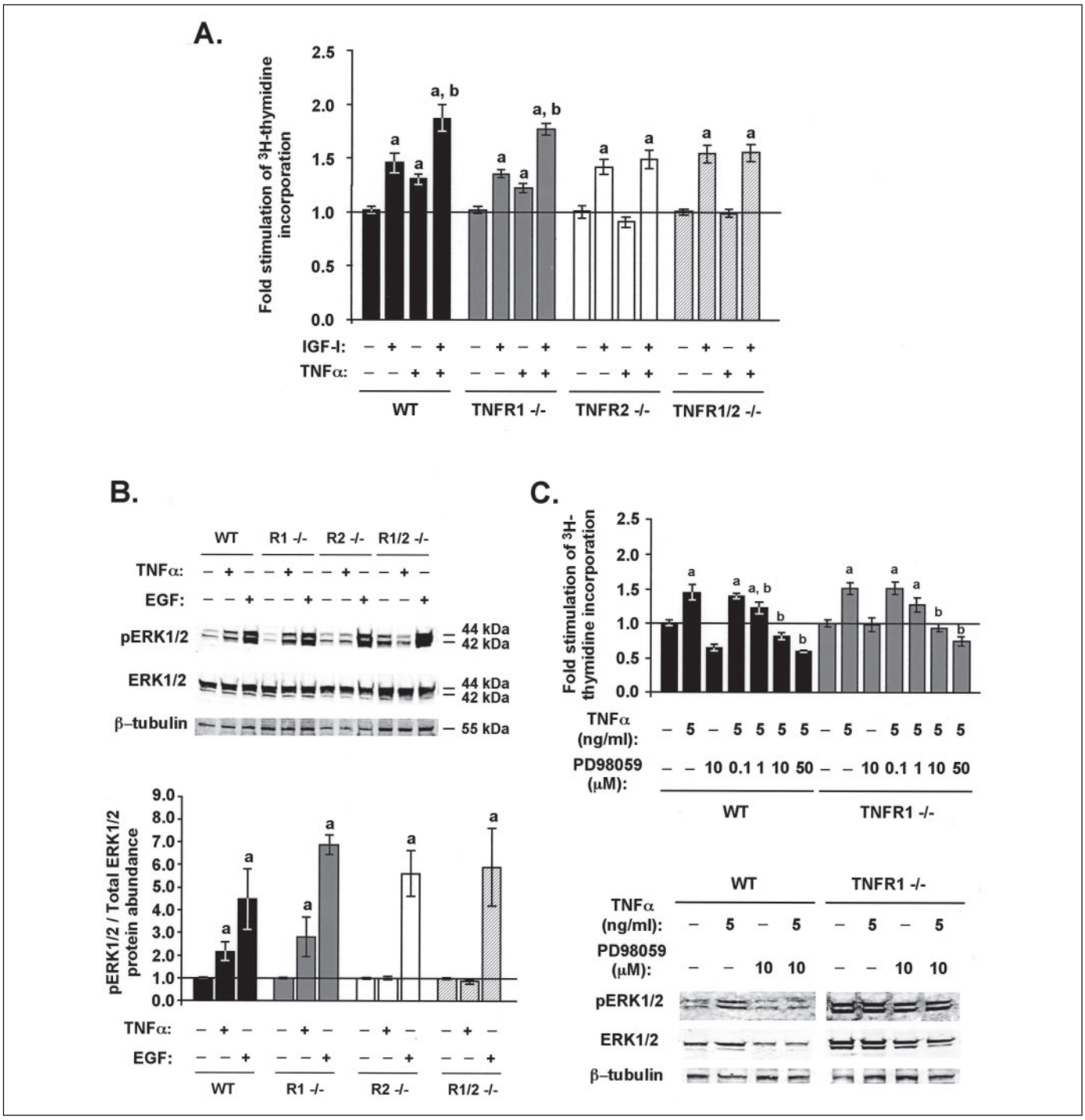

FIGURE 3. **TNFR2 and ERK1/2 activation are essential for TNFα-stimulated DNA synthesis in intestinal myofibroblasts.** *A*, -fold stimulation of [^{3}H]thymidine incorporation into DNA by IGF-I (10 ng/ml) and TNFα (5 ng/ml) alone or in combination. *a*, $p < 0.05$ *versus* no treatment; *b*, $p < 0.05$ *versus* IGF-I or TNFα alone. n = four separate experiments done in triplicate. *B*, representative Western immunoblots showing pERK1/2, total ERK1/2, and control β-tubulin protein from total protein isolated from cells treated with TNFα (5 ng/ml) or EGF (10 ng/ml; positive control) for 30 min. Histograms show mean ± S.E. *a*, $p < 0.05$ *versus* no treatment; $n = 4$ per treatment group. *C*, TNFα induction of [^{3}H]thymidine incorporation into DNA of WT and TNFR1$^{-/-}$ cells is inhibited in a dose-dependent manner by PD98059, an ERK1/2 inhibitor. *a*, $p < 0.05$ *versus* no treatment; *b*, $p < 0.05$ *versus* TNFα; $n = 6$ per treatment. TNFα-induced activation of ERK1/2 is inhibited by PD98059 as shown in the representative Western immunoblots.

alterations in collagen protein, TIMP-1 mRNA abundance was assayed from total RNA isolated from cultured intestinal myofibroblasts treated with IGF-I, TNFα, or IGF-I + TNFα. We assayed TIMP-1 because it is increased during Crohn's disease and in animal models of intestinal inflammation (43–46). TNFα, but not IGF-I, caused a 2.5-fold induction of TIMP-1 mRNA expression (Fig. 1*D*). Effects of TNFα given in combination with IGF-I on TIMP-1 mRNA were similar to those observed with TNFα alone (Fig. 1*D*).

TNFα Decreases Collagen Degradation and MMP-2 Activity in Intestinal Myofibroblasts—Compared with no treatment controls, TNFα treatment caused no change in the rate of collagen synthesis, as measured by [^{3}H]proline incorporation into collagen protein (Fig. 2*A*). Compared with untreated controls, TNFα treatment decreased the rate of collagen degradation during the chase with cold proline, which was particularly evident at early time points during the chase with cold proline, resulting in less collagen degradation in the sam-

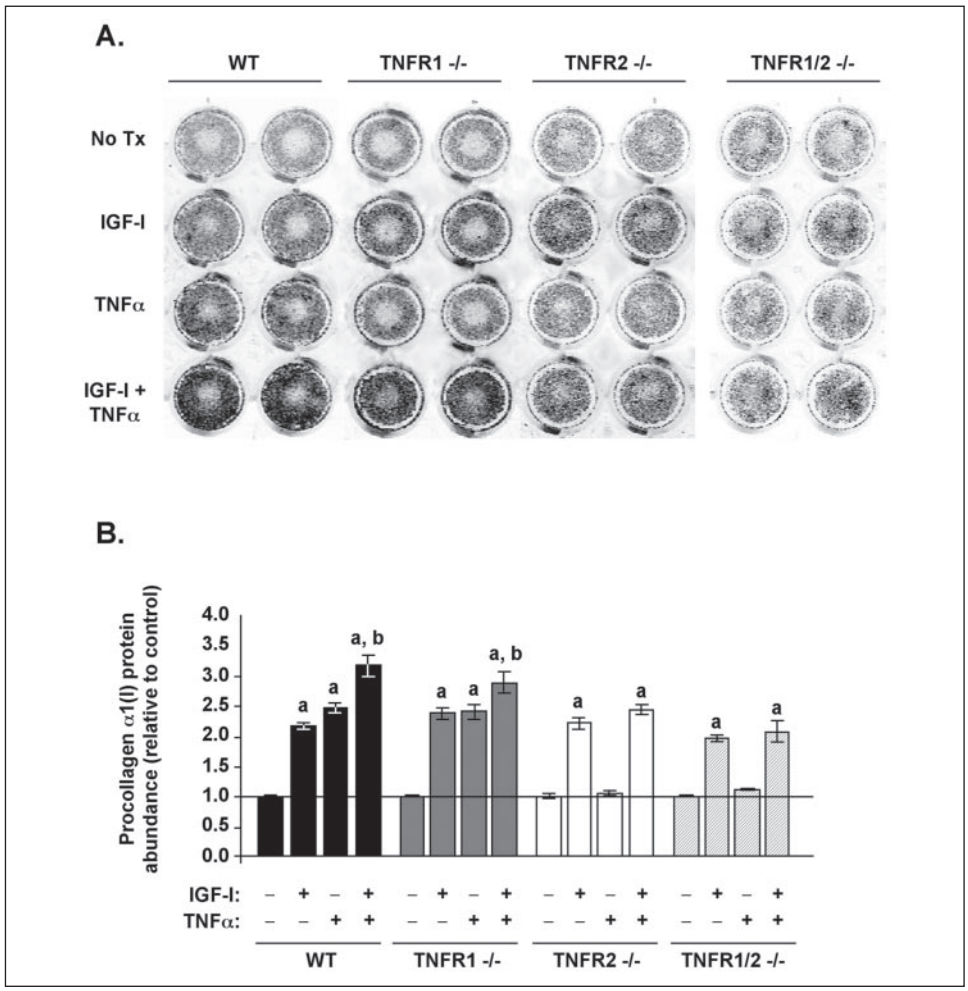

FIGURE 4. **TNFR2 is essential for TNFα induction of collagen protein accumulation.** *A*, representative in-cell Western blot showing WT, TNFR1$^{-/-}$, TNFR2$^{-/-}$, or TNFR1/2$^{-/-}$ myofibroblasts plated in an equal number in a 24-well culture plate and treated with IGF-I (10 ng/ml) and TNFα (5 ng/ml) alone or in combination. *B*, histograms showing mean ± S.E. of the -fold induction relative to no treatment controls of the same genotype. *a*, $p < 0.05$ *versus* no treatment (*No Tx*); *b*, $p < 0.05$ *versus* IGF-I or TNFα alone. $n \geq$ three separate experiments done in duplicate.

ples treated with TNFα compared with no treatment at the end of a 24-h chase period.

Gelatinolytic activity of conditioned media from intestinal myofibroblasts treated with TNFα was assessed by gelatin zymography. Gelatinolytic bands of 62 kDa corresponding to MMP-2 activity were decreased in a dose-dependent manner by TNFα (Fig. 2*B*). Activity of MMP-9 was variable and showed no significant change with TNFα treatment (data not shown).

TNFR2 and ERK1/2 Activation Are Essential for TNFα-stimulated DNA Synthesis in Intestinal Myofibroblasts—Intestinal myofibroblasts isolated from ileum of WT, TNFR1$^{-/-}$, TNFR2$^{-/-}$, and TNFR1/2$^{-/-}$ mice were used to determine which TNF receptor mediates TNFα induction of DNA synthesis. TNFR1/2$^{-/-}$ myofibroblasts were used as a negative control because they are deficient in TNFα signaling. IGF-I and TNFα alone increased DNA synthesis and showed an additive response in WT and TNFR1$^{-/-}$ myofibroblasts when given in combination (Fig. 3*A*). In TNFR2$^{-/-}$ and TNFR1/2$^{-/-}$ cells, IGF-I but not TNFα increased DNA synthesis. In TNFR2$^{-/-}$ and TNFR1/2$^{-/-}$ cells, effects of IGF-I and TNFα given in combination on DNA synthesis were similar to that of IGF-I treatment alone (Fig. 3*A*). Collectively, these results indicate that TNFR2 is essential for TNFα-induced DNA synthesis in intestinal myofibroblasts.

Western immunoblot analyses revealed activation of ERK1/2 by TNFα and EGF, which was used as a positive control. The effect of TNFα was absent in TNFR2$^{-/-}$ and TNFR1/2$^{-/-}$ cells, but was retained in TNFR1$^{-/-}$ cells (Fig. 3*B*). Induction of p38 MAPK and c-Jun NH_2-terminal kinase was unaffected by TNFR2$^{-/-}$ deletion (data not shown). To determine whether TNFα-induced cell proliferation, as measured by DNA synthesis, is dependent on ERK1/2 signaling, WT and TNFR1$^{-/-}$ cells were treated with PD98059, an ERK1/2-specific inhibitor, for 30 min prior to treatment with TNFα. TNFα-stimulated [^{3}H]thymidine incorporation into DNA in WT and TNFR1$^{-/-}$ cells in the absence of PD98059 (Fig. 3*C*). PD98059 inhibited TNFα-stimulated [^{3}H]thymidine incorporation into DNA in a dose-dependent manner (Fig. 3*C*). PD98059 inhibited TNFα-stimulated ERK1/2 activation as shown by Western immunoblot from protein extracts isolated from WT and TNFR1$^{-/-}$ cells treated in replicate with the [^{3}H]thymidine incorporation assay (Fig. 3*C*, *bottom panel*). These results indicate that TNFα-induced DNA synthesis in intestinal myofibroblasts is dependent on ERK1/2 signaling.

TNFR2 Is Essential for TNFα Induction of Collagen Protein Accumulation—Intestinal myofibroblasts isolated from ileum of WT, TNFR1$^{-/-}$, TNFR2$^{-/-}$, and TNFR1/2$^{-/-}$ mice were used to determine whether TNFR2 also mediates TNFα-induced procollagen α1(I) protein accumulation in these cells. IGF-I and TNFα increased procollagen α1(I) protein in WT and TNFR1$^{-/-}$ myofibroblasts (Fig. 4). IGF-I, but not TNFα, increased procollagen α1(I) protein in TNFR2$^{-/-}$ and TNFR1/2$^{-/-}$ cells. Similarly, IGF-I and TNFα had an additive effect on collagen accumulation in WT and TNFR1$^{-/-}$ cells, but the response in TNFR2$^{-/-}$ and TNFR1/2$^{-/-}$ cells was similar to that of IGF-I treatment alone (Fig. 4). Subsequent staining with β-tubulin ensured even plating of cells across wells (data not shown). Collectively, these results indicate

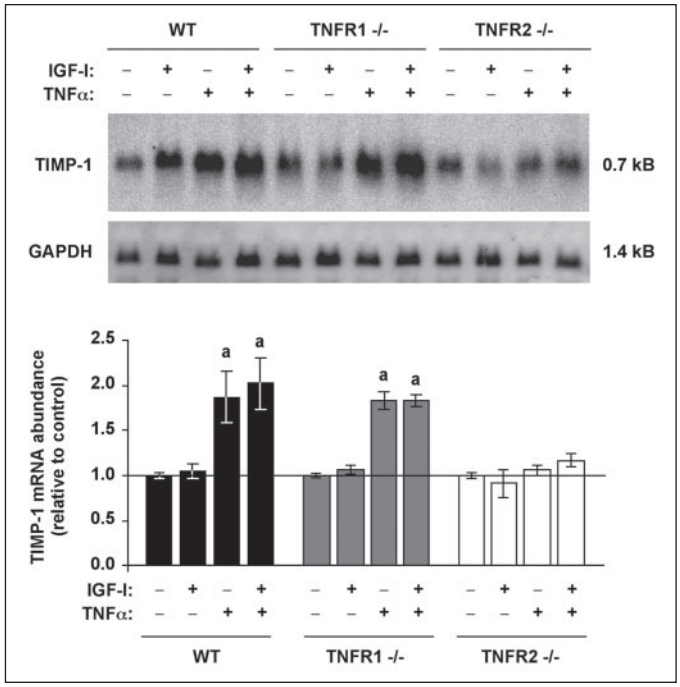

FIGURE 5. **TNFα-induced TIMP-1 mRNA expression is mediated by TNFR2.** Representative Northern blots showing TIMP-1 and GAPDH mRNA abundance in total RNA isolated from WT, TNFR1$^{-/-}$, and TNFR2$^{-/-}$ myofibroblasts treated with IGF-I (10 ng/ml), TNFα (5 ng/ml), or IGF-I + TNFα. Histograms showing mean ± S.E. of the -fold difference relative to no treatment controls of the same genotype. *a*, $p < 0.05$ *versus* no treatment. $n = 4$ per treatment group.

that TNFR2 is essential for TNFα action on collagen protein accumulation in intestinal myofibroblasts.

TNFα-induced TIMP-1 mRNA Expression Is Mediated by TNFR2—To determine whether the diminished effect of TNFα on collagen protein accumulation in TNFR2$^{-/-}$ myofibroblasts was linked to attenuated TIMP-1 induction, TIMP-1 mRNA abundance was measured in WT, TNFR1$^{-/-}$, and TNFR2$^{-/-}$ cells. TNFα, alone or in combination with IGF-I, increased TIMP-1 expression in WT and TNFR1$^{-/-}$ myofibroblasts but no significant induction of TIMP-1 was observed in TNFR2$^{-/-}$ cells (Fig. 5). Therefore, TNFR2 is necessary for TNFα-mediated induction of TIMP-1 mRNA as well as collagen accumulation in intestinal myofibroblasts.

NFκB or AP-1 Pathways Do Not Account for Diminished Collagen Accumulation in TNFR2$^{-/-}$ Cells—Collagen accumulation stimulated by TNFα was not affected by PD98059, an ERK1/2-specific inhibitor, as measured by in-cell Western blots (data not shown), which suggested that TNFα-induced collagen accumulation, in contrast to proliferation, is not dependent on ERK1/2 signaling. Transcriptional activation of NFκB and AP-1 was therefore assessed because both NFκB and AP-1 are the two major transcription factors activated by TNFα (47). Intestinal myofibroblasts were infected with an adenovirus expressing an NFκB (Ad.NFκBLuc) or AP-1 (Ad.AP-1Luc) responsive luciferase reporter gene and treated with TNFα, IGF-I, or TNFα and IGF-I in combination. WT myofibroblasts show increased NFκB transcriptional activation with TNFα treatment but no effect by IGF-I treatment (Fig. 6*A*). TNFR1$^{-/-}$ and TNFR2$^{-/-}$ myofibroblasts show increased NFκB transcriptional activation by TNFα only at the higher dose, but this response is diminished compared with WT cells (Fig. 6*A*). As a negative control, TNFR1/2$^{-/-}$ myofibroblasts show complete ablation of NFκB transcriptional activity by TNFα. Cells treated with TNFα and IGF-I in combination show similar NFκB transcriptional activation as TNFα treatment alone. Collectively, these results suggest in the absence of either TNF receptor, that TNFα-induced transcriptional activity of NFκB is reduced compared with WT. However, because only myofibroblasts lacking functional TNFR2 but not cells lacking functional TNFR1 show diminished TNFα-induced collagen protein and TIMP-1 mRNA, the diminished NFκB activation cannot account for this difference between TNFR1$^{-/-}$ and TNFR2$^{-/-}$ cells.

As shown in Fig. 6*B*, WT cells show increased AP-1 transcriptional activity when treated with IGF-I and the higher dose of TNFα. TNFR1$^{-/-}$ and TNFR2$^{-/-}$ myofibroblasts show similar activation of AP-1 transcription by TNFα and IGF-I as WT cells, indicating the functional loss of one TNF receptor can be compensated for by the other TNF receptor in terms of AP-1 transcriptional activity. TNFR1/2$^{-/-}$ myofibroblasts showed similar activation of AP-1 transcription by IGF-I as the other genotypes, but showed no induction of AP-1 transcription by TNFα, as expected because these cells are devoid of TNFα signaling (Fig. 6*B*). Collectively, these results suggest that diminished TNFα-induced collagen protein accumulation and TIMP-1 expression in TNFR2$^{-/-}$ myofibroblasts compared with WT and TNFR1$^{-/-}$ cells are not associated with decreased AP-1 transcriptional activation.

STAT Activation Is Altered in TNFR2$^{-/-}$ Cells and Expression of Constitutively Active STAT3 Is Sufficient to Rescue TNFα-induced TIMP-1 Expression in TNFR2$^{-/-}$ Cells—Emerging evidence in other cell types indicate cross-talk between TNFα-activated pathways and JAK-STAT pathways (48–50). Electromobility shift assays using a radiolabeled consensus DNA binding site for STAT3 showed that TNFα induced STAT3 response element binding activity in WT and TNFR1$^{-/-}$ cells (Fig. 7*A*). In TNFR2$^{-/-}$ cells, TNFα also induced binding to the STAT3 consensus sequence but the mobility of this complex was altered compared with WT and TNFR1$^{-/-}$ cells. This binding could be competed with unlabeled STAT3 oligonucleotides but not STAT1 (Fig. 7*A*) or STAT5 oligonucleotides (data not shown). Because the promoter of the TIMP-1 gene, which is induced by TNFα in WT and TNFR1$^{-/-}$ cells but not TNFR2$^{-/-}$ cells, contains a nonconsensus binding site for STAT3 (41), electromobility shift assays were also performed using oligonucleotides corresponding to this binding site. TNFα induced nonconsensus STAT3 response element binding activity in WT cells but not in TNFR2$^{-/-}$ cells (Fig. 7*B*), suggesting that in TNFR2$^{-/-}$ cells STAT3 binding to the STAT3 response element in the TIMP-1 promoter is impaired.

To assess directly if STAT3 plays a role in TIMP-1 induction, TNFR2$^{-/-}$ cells were transfected with a constitutively active form of STAT3 or a control GFP expressing adenovirus. As in previous experiments, TNFα failed to induce TIMP-1 in TNFR2$^{-/-}$ cells transfected with the control GFP adenovirus (Fig. 7*C*). However, STAT3 adenovirus infection induced TIMP-1 expression in TNFR2$^{-/-}$ cells regardless of whether cells were treated with TNFα (Fig. 7*C*). This suggests that activated STAT3 is sufficient to induce TIMP-1 in TNFR2$^{-/-}$ cells.

DISCUSSION

TNFα is a well established mediator of chronic inflammatory diseases including Crohn's disease. The role of TNFα in inflammation-induced intestinal fibrosis is not well defined, which could be due, at least in part, to the difficulty of segregating direct *in vivo* effects of TNFα on fibrosis from secondary effects of TNFα on inflammation. Additionally, very few mouse models of intestinal inflammation are accompanied with fibrosis. For this reason, this study used cultured intestinal myofibroblasts, cell types thought to mediate fibrosis (2, 51, 52), as a model system to study direct actions of TNFα. Our findings provide novel information that TNFα induces collagen accumulation and has mitogenic effects in intestinal myofibroblasts, providing important evidence

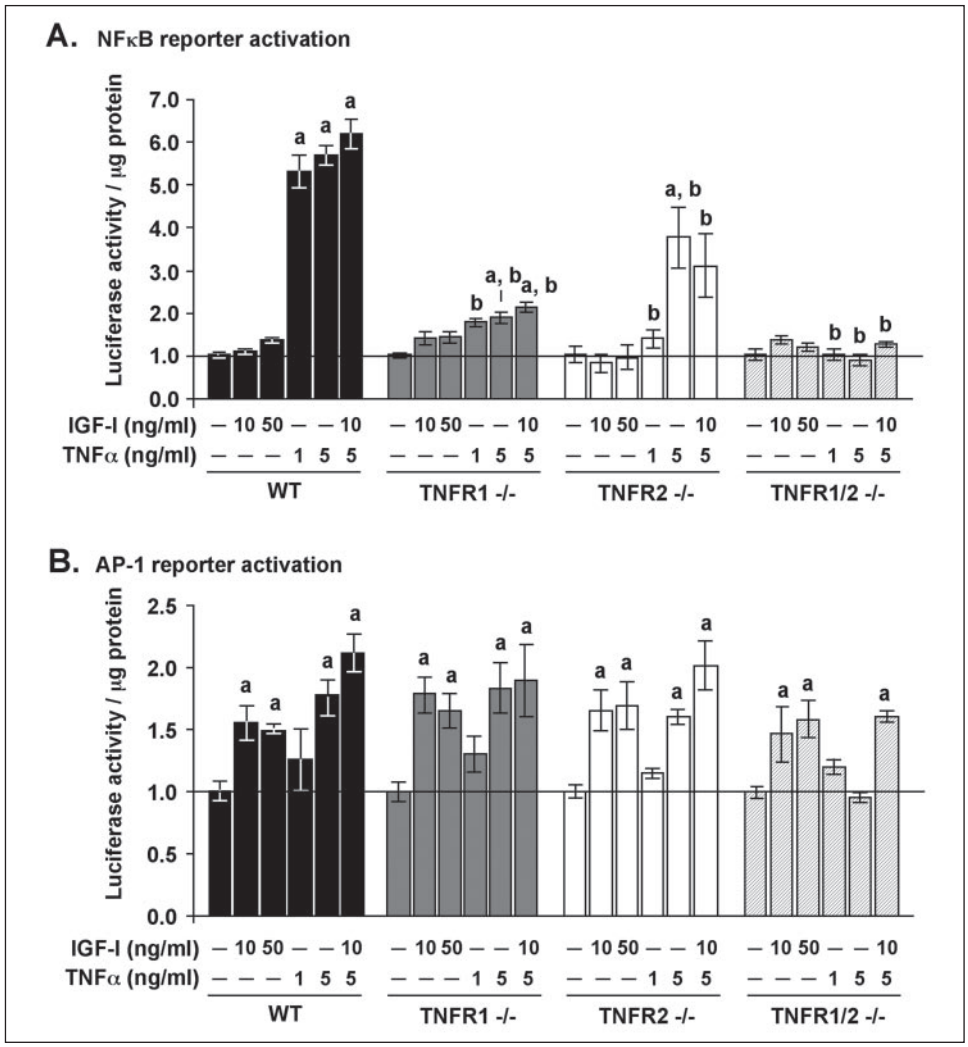

FIGURE 6. **Diminished collagen accumulation in TNFR2$^{-/-}$ cells is not associated with alterations in NFκB or AP-1 transcriptional activation.** Intestinal myofibroblasts were infected with Ad.NFκBLuc virus (*A*) or Ad.AP-1Luc virus (*B*) and treated for 8 h with increasing concentrations of TNFα or IGF-I (ng/ml) as indicated *below* each graph. Histograms showing mean ± S.E. of the -fold difference relative to no treatment controls of the same genotype. *a*, $p < 0.05$ *versus* no treatment; *b*, $p < 0.05$ *versus* WT of same treatment. $n = 6$ for WT, TNFR1$^{-/-}$, and TNFR2$^{-/-}$. $n = 3$ for TNFR1/2$^{-/-}$.

for pro-fibrogenic actions of TNFα in intestine. TNFα also had additive effects on collagen accumulation and myofibroblast proliferation when combined with IGF-I, a growth factor implicated as a key pro-fibrogenic mediator during intestinal inflammation *in vivo* (2, 22, 23, 26). Our studies reveal that additive effects of IGF-I and TNFα on total collagen accumulation are mediated by IGF-I induction of collagen gene transcription and TNFα-induced inhibition of collagen degradation via TIMP-1. Furthermore, IGF-I and TNFα additively stimulate intestinal myofibroblast DNA synthesis, a measure of cell proliferation. Because these cell types are known to produce collagen, expansion of myofibroblasts could also promote fibrosis.

TNFR2 is emerging as a key receptor that is up-regulated in experimental and clinical inflammatory bowel disease (18, 19). Our findings provide new information that TNFR2 is essential for TNFα induction of DNA synthesis and collagen protein accumulation in intestinal myofibroblasts because TNFR2$^{-/-}$ and TNFR1/2$^{-/-}$ cells showed no response to TNFα compared with WT and TNFR1$^{-/-}$ cells. The loss of TNFα-induced collagen accumulation in TNFR2$^{-/-}$ cells is associated with impaired TIMP-1 expression. Importantly, TNFR2$^{-/-}$ cells showed similar stimulation of collagen accumulation and DNA synthesis in response to IGF-I as WT and TNFR1$^{-/-}$ cells indicating the lack of response was specific to TNFα. Collectively, these results suggest that TNFR2 rather than TNFR1 is the primary mediator of the pro-fibrotic effects of TNFα in intestinal myofibroblasts.

Much effort has focused on the signaling pathways modulating the proinflammatory and proapoptotic or anti-apoptotic actions of TNFα. Our studies provide novel information about TNFR1- and TNFR2-linked pathways leading to increased collagen accumulation and proliferation in intestinal myofibroblasts. ERK1/2 activation was absent in TNFR2$^{-/-}$ cells. Furthermore, the TNFα-induced cell proliferation, but not collagen accumulation, in WT and TNFR1$^{-/-}$ myofibroblasts was blocked by an ERK1/2-specific inhibitor. These results suggest that TNFα-stimulated cell proliferation is dependent on TNFR2 and ERK1/2 signaling in intestinal myofibroblasts. Luciferase reporter assays in WT, TNFR1$^{-/-}$, TNFR2$^{-/-}$, and TNFR1/2$^{-/-}$ cells indicate that both TNF receptor isoforms activate NFκB and AP-1, two major transcription factors known to be activated by TNFα in other cell types (47). However, the substantial NFκB and AP-1 activation remaining in TNFR2$^{-/-}$ cells is clearly insufficient to mediate the collagen and TIMP-1 inducing effects of TNFα because these responses were absent in TNFR2$^{-/-}$ cells. Furthermore, NFκB activation by TNFα was greatly attenuated in TNFR1$^{-/-}$ cells, yet these cells show preserved collagen accumulation and TIMP-1 induction by TNFα. Examination of other pathways revealed that in TNFR2$^{-/-}$ cells TNFα activated the binding of nuclear proteins to a STAT3 consensus response element, but the complex differed from the STAT3 binding complex in WT or TNFR1$^{-/-}$ cells. Moreover, TNFα-induced binding of nuclear proteins to a STAT3 nonconsensus response element located in the TIMP-1

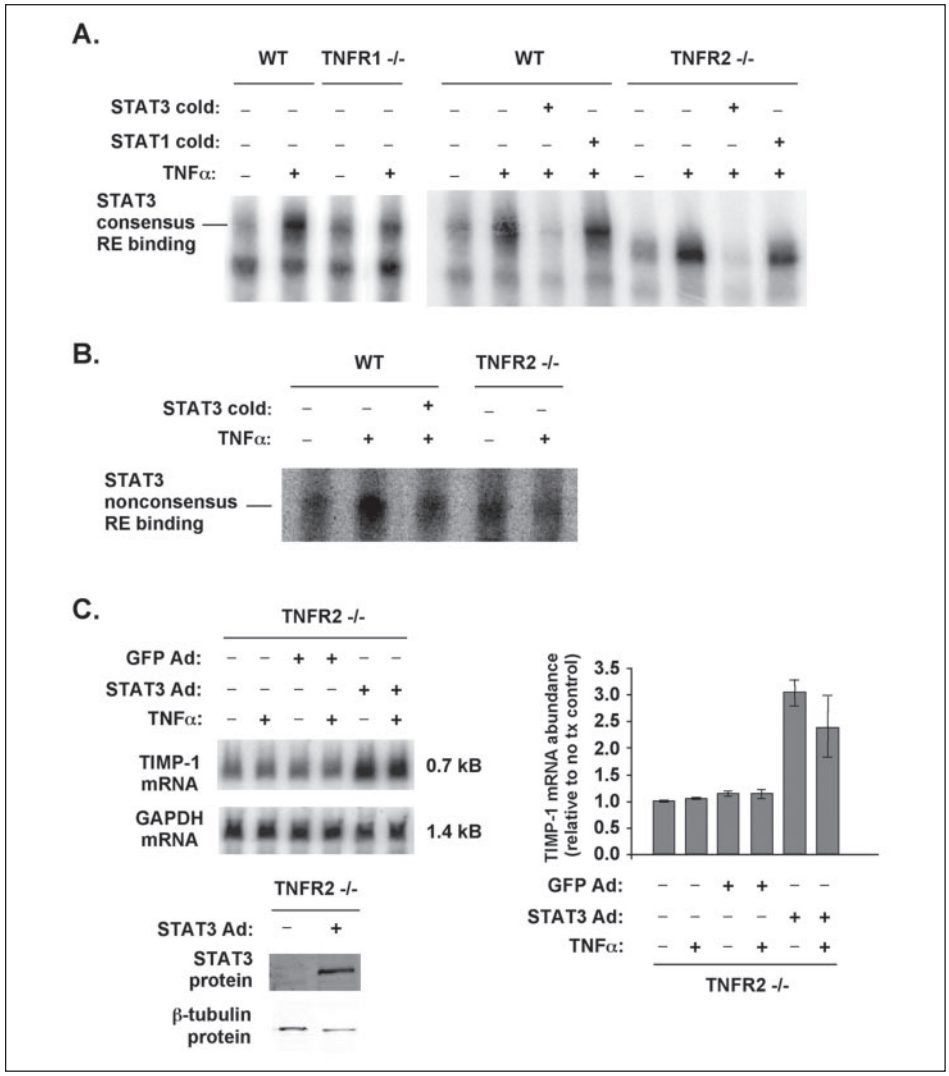

FIGURE 7. **TNFα-induced STAT3 response element binding is altered in TNFR2$^{-/-}$ cells and constitutively active STAT3 induces TIMP-1 in TNFR2$^{-/-}$ cells.** *A*, electromobility shift assay showing binding of nuclear proteins from WT, TNFR1$^{-/-}$, and TNFR2$^{-/-}$ cells treated with 5 ng/ml TNFα to a STAT3 consensus response element (*RE*). Binding is competed by unlabeled STAT3 oligonucleotides (STAT3 cold) but not STAT1 oligonucleotides (STAT1 cold). The mobility of the binding complex in TNFR2$^{-/-}$ cells is altered. *B*, electromobility shift assay showing binding of nuclear proteins from WT but not TNFR2$^{-/-}$ cells treated with 5 ng/ml TNFα to the STAT3 nonconsensus response element located in the TIMP-1 promoter. *C*, representative Northern blot showing TIMP-1 and GAPDH mRNA abundance in total RNA isolated from TNFR2$^{-/-}$ myofibroblasts infected with constitutively active STAT3 (*STAT3 Ad*) or GFP expressing adenovirus ± TNFα (5 ng/ml). Histograms show mean ± S.E. of -fold induction relative to untreated controls. Western immunoblot showing overexpression of STAT3 in TNFR2$^{-/-}$ cells infected with constitutively active STAT3 adenovirus.

promoter (41) in WT cells but this response was absent in TNFR2$^{-/-}$ cells. Expression of constitutively active STAT3 in TNFR2$^{-/-}$ cells was sufficient to induce TIMP-1 expression. Together these findings indicate that TNFR2 acting via STAT3 rather than the NFκB or AP-1 pathways typically associated with TNFR action is required for and sufficient to induce TIMP-1, which appears to be the major mediator of TNFα-induced collagen accumulation. Whereas our data demonstrate that STAT3 can induce TIMP-1 expression in intestinal myofibroblasts and that TNF stimulates binding to the STAT3 response element in the TIMP-1 promoter, our studies suggest that more detailed analyses of the mechanisms by which different TNF receptors link to STAT pathways will be of interest in the future. This is because the different mobility of protein-DNA complexes binding to a STAT3 consensus element in TNFR2$^{-/-}$ cells *versus* WT and TNFR1$^{-/-}$ cells suggests that TNFR1 and TNFR2 may couple to different STAT isoforms. Future studies of whether distinct STAT-coupled pathways are activated by TNFR1 *versus* TNFR2 will therefore be of interest.

In summary, our study provides new evidence for additive interactions between TNFα and IGF-I on collagen accumulation in intestinal myofibroblasts and defines the mechanisms of action as depicted in Fig. 8. Our data suggest that IGF-I promotes fibrosis by primary effects on collagen gene transcription to increase collagen synthesis and by stimulating expansion of fibrogenic cells. TNFα, acting primarily via

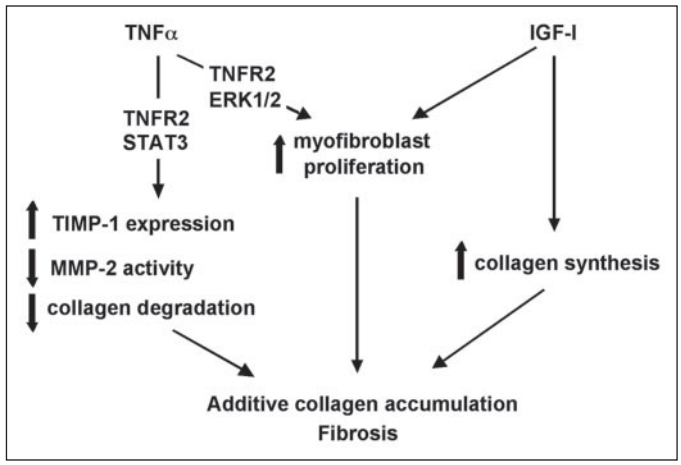

FIGURE 8. **Hypothetical model of TNFα and IGF-I actions leading to increased collagen accumulation.** IGF-I promotes expansion of fibrogenic intestinal myofibroblasts and stimulates collagen gene transcription to increase collagen synthesis. TNFα, acting primarily via TNFR2, promotes fibrosis by stimulating myofibroblast proliferation in an ERK-dependent manner and reducing collagen degradation by inducing TIMP-1 via a STAT3-dependent mechanism. Through these distinct mechanisms TNFα and IGF-I additively increase collagen accumulation.

TNFR2, promotes fibrosis by stimulating myofibroblast proliferation in an ERK-dependent manner and reducing collagen degradation by inducing TIMP-1 via STAT3. Importantly, the distinct mechanisms by which TNFα and IGF-I promote collagen accumulation result in an additive increase in collagen accumulation. This suggests that therapies targeted specifically at limiting TNFR2 actions and combined with inhibitors of IGF-I action may hold promise for limiting fibrosis associated with intestinal inflammation and Crohn's disease, for which there are currently no approved or effective pharmacological therapies. Our findings of additive interactions of TNFα and IGF-I on collagen accumulation in intestinal myofibroblasts also indicate that such interactions may be relevant to mechanisms of fibrosis and therapy development in other organ systems.

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