The proinflammatory cytokine, TNFα plays a major role in muscle wasting occurring in chronic diseases and muscular dystrophies. Among its other functions, TNFα perturbs muscle regeneration by preventing satellite cell differentiation. In the present study, the role of c-Jun N-terminal kinase (JNK), a mediator of TNFα, was investigated in differentiating myoblast cell lines. Addition of TNFα to C2 myoblasts induced immediate and delayed phases of JNK activity. The delayed phase is associated with myoblast proliferation. Inhibition of JNK activity prevented proliferation and restored differentiation to TNFα-treated myoblasts. Studies with cell lines expressing MyoD:ER chimera and lacking JNK1 or JNK2 genes indicate that JNK1 activity mediates the effects of TNFα on myoblast proliferation and differentiation. TNFα does not induce proliferation or inhibit differentiation of JNK1-null myoblasts. However, differentiation of JNK1-null myoblasts is inhibited when they are grown in conditioned medium derived from cell lines affected by TNFα. We investigated the induced synthesis of several candidate growth factors and cytokines following treatment with TNFα. Expression of IL-6 and leukemia inhibitory factor (LIF) was induced by TNFα in wild-type and JNK2-null myoblasts. However, LIF expression was not induced by TNFα in JNK1-null myoblasts. Addition of LIF to the growth medium of JNK1-null myoblasts prevented their differentiation. Moreover, LIF-neutralizing antibodies added to the medium of C2 myoblasts prevented inhibition of differentiation mediated by TNFα. Hence, TNFα promotes myoblast proliferation through JNK1 and prevents myoblast differentiation through JNK1-mediated secretion of LIF.

Massive loss of skeletal muscle occurs in a variety of disorders: chronic catabolic conditions such as AIDS, sepsis, and cancer and genetic disorders, collectively known as muscular dystrophies. In all cases of muscle wasting, including chronic diseases or genetic disorders, it is believed that inflammation contributes to the process (1). Neutrophiles and macrophages invade the damaged muscle tissue and secrete proinflammatory cytokines (2). Chronic secretion of proinflammatory cytokines, tumor necrosis factor α (TNFα)2 in particular, has been found to actively damage the muscle tissue. In fact, blocking TNFα function significantly reduces the atrophy of skeletal muscle (3). TNFα is involved in many of the events leading to muscle wasting: inhibition of the differentiation process, inhibition of protein synthesis, selective proteolysis, and activation of caspase-mediated apoptosis (4, 5). Thus, muscle loss is most probably a result of the perturbed balance between muscle degeneration and regeneration caused by proinflammatory cytokines.

Muscle regeneration occurs by the expansion and differentiation of satellite cells that lie beneath the basal lamina (6). Normally these cells are quiescent (resting), expressing the paired box transcription factor Pax7, but not the myogenic regulatory factors (MRFs). In response to trauma, such as injury, these cells are activated, first by initiating the expression of MyoD and then by proliferation. Activation of satellite cells is promoted by the release of growth factors such as hepatocyte growth factor (HGF), fibroblast growth factors (FGFs), and cytokines like IL-6 from the injured tissue and infiltrating macrophages. Finally, the expression of myogenin is induced, and cells differentiate and fuse with preexisting myofibers (reviewed in Ref. 7). Perturbation of muscle regeneration at any stage: activation, proliferation, or differentiation, causes fatal loss of muscle tissue. TNFα perturbs muscle regeneration in chronic and genetic disorders, though the mechanism is still obscure (8).

TNFα affects several intracellular signaling pathways leading to the activation of NFκB, caspase 8, and stress-induced pathways like c-Jun N-terminal kinase (JNK) and p38 MAPK (9). Whereas the involvement of NFκB and caspases in muscle wasting has been extensively studied, relatively little is known about the role of stress pathways. Several studies have indicated that NFκB activity inhibits muscle differentiation by reducing

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1 The on-line version of this article (available at http://www.jbc.org) contains supplemental Figs. S1–S5.

2 To whom correspondence should be addressed: PO Box 9649, Haifa 31096, Israel. Tel: 972-4-8295-287; Fax: 972-4-8553-299; E mail: bengal@tx.technion.ac.il.

3 The abbreviations used are: TNFα, tumor necrosis factor α; JNK, c-Jun N-terminal kinase; LIF, leukemia inhibitory factor; ER, estrogen receptor; NFκB, nuclear factor κB; MyHC, myosin heavy chain; BrdU, bromodeoxyuridine; DM, differentiation medium; PBS, phosphate-buffered saline; FACS, fluorescence-activated cell sorting; IL, interleukin; DAPI, 4′,6-diamidino-2-phenylindole; RT, reverse transcriptase.
the levels of the MyoD protein and activating the expression of cyclin D1 (10–12). The role of JNK in the effect of TNFα on myogenesis has been less investigated. A recent study suggested that activation of JNK by TNFα blocks IGF-1 signaling necessary for the differentiation of myoblasts (13). Yet, the mechanism has not been fully explored; is JNK involved in myoblast proliferation, or alternatively is JNK involved in inhibition of myoblast differentiation but not in proliferation? Which isoforms of JNK participate in these processes? How does JNK affect these processes?

In the present work we approached these questions. We have shown that TNFα induces proliferation via JNK and indirectly prevents differentiation of myoblasts. The JNK1 isoform is involved in these processes. Interestingly, JNK1 mediates the expression of LIF, which prevents the differentiation process independently of proliferation. Therefore, our results indicate that TNFα affects myoblast proliferation directly via JNK1 and myoblast differentiation indirectly through the autocrine effect of LIF.

**EXPERIMENTAL PROCEDURES**

**Materials**—SP600125 (JNK inhibitor) was purchased from BIOMOL, dissolved in DMSO, and added to the culture medium to a final concentration of 20 µM. TNFα was purchased from Cytolabs, was dissolved in water, and added to the culture medium to a final concentration of 20 ng/ml. LIF was purchased from Calbiochem and was added to cell culture to a final concentration of 20 ng/ml. Cytosine arabinoside (Ara-C) was purchased from Calbiochem, dissolved in PBS, and added to culture medium to a final concentration of 100 µM. Thymidine was added to cell medium at a concentration of 2 mM. Neutralizing LIF antibody was from Chemicon and was added at 1 µg/ml.

**Cell Culture**—C2 cells were a gift from Dr. David Yaffe (14). Cell lines were maintained in Dulbecco’s modified Eagle’s medium supplemented with 15% calf serum (HyClone), penicillin, and streptomycin (growth medium, GM). To induce differentiation, we used Dulbecco’s modified Eagle’s medium supplemented with 10 µg of insulin per ml and 10 µg of transferrin per ml (differentiation medium, DM). Fibroblasts from different mice strains (wild type, JNK1−/−, JNK2−/−, c-Jun−/−, and IKKβ−/−) were infected with a retrovirus encoding the MyoD protein and the hormone binding domain of estrogen receptor (pBABE puro MyoD:ER) (15). Myoblast cell lines were isolated following selection with puromycin (3 µg/ml). These cells were grown in Dulbecco’s modified Eagle’s medium without phenol red supplemented with 15% calf serum (GM). Addition of 10−7 m β-estradiol to DM induced translocation of the cytoplasmic chimera protein into the nucleus and initiation of the myogenic program (supplemental Fig. S1). In some experiments, conditioned media from certain cell lines (wild-type, JNK1−/−, and JNK2−/− MyoD:ER) were used to replace the medium of JNK1−/− MyoD:ER cells grown in parallel. The medium was replaced every 6 h with fresh conditioned medium of growing cells.

**Western Analysis**—Cells were lysed, whole cell extracts were collected, and equal amounts of extracted proteins (40 µg) were loaded and separated over 10% SDS-PAGE and transferred to nitrocellulose membranes. Membranes were incubated in blocking buffer (20 mM Tris-HCl, pH 7.4, 150 mM NaCl, 0.1% Tween 20, 5% w/v nonfat dry milk) and then in the same buffer with the primary and secondary antibodies. Between the second and third incubations, membranes were washed three times in 0.1% Tween 20 and 1× TBS (20 mM Tris-HCl, pH 7.4, 150 mM NaCl). Immunoblotting was conducted with the following antibodies: anti-c-Jun and anti-phospho-c-Jun (Santa Cruz Biotechnology) 1:1000, anti-myogenin, F5D (1:1000) and anti-MyHC, MF20 (1:1000), anti-JNK and anti-phospho-JNK, anti-α-tubulin (Sigma Aldrich) 1:10,000. Proteins were visualized using the enhanced chemiluminescence kit of Pierce.

**Immunohistochemical Staining**—Cells were fixed and immunostained. The primary antibodies used were monoclonal anti-MyHC (MF-20), anti-pc-Jun (Santa Cruz Biotechnology), monoclonal anti-phospho-histone H3 (Upstate Biotechnology), and anti-BrdU. Cells were exposed to secondary antibody and DAPI. The immunochemically stained cells were viewed at ×200 or ×650 magnification under a fluorescence microscope (Olympus, model BX50) and photographed with a digital camera.

**Cell Cycle Analysis**—BrdU was added to cell medium to a final concentration of 10 µM. After 3 h, the cells were washed with PBS, fixed with methanol, and permeabilized in 0.25% Triton X–100. Following a PBS wash, the cells were incubated in 2 N HCL solution for 1 h. The solution was neutralized by washing the cells with 0.1 M borate buffer (pH 8.5). The cells were incubated for 90 min with 6 mg/ml anti-BrdU antibody (BMC9318, Roche Applied Science) in PBS containing 5% bovine serum albumin. Cells were exposed to a secondary antibody and DAPI and finally viewed at ×200 magnification under a fluorescence microscope (Olympus, model BX50).

Cells that were analyzed by fluorescence-activated cell sorting (FACS) were washed twice with PBS, fixed with 70% ethanol and treated with 2 mg/ml of RNase A (Worthington, Lakewood, NJ) for 30 min at 37 °C. Ten minutes before analysis, propidium iodide (PI) was added at 100 µg/ml. DNA content was analyzed by using FACS and ModFit software (Becton Dickinson).

**RT-PCR (Reverse Transcriptase PCR)**—After removal of the culture medium, the cells were washed twice with ice-cold PBS. Total RNA extraction was performed using a commercial kit, TRI REAGENT™ (MRC). The procedure was conducted according to the manufacturer’s protocol. RT reactions were performed following DNaseI treatment, phenol-chloroform extraction, and ammonium acetate precipitation. RNA was incubated with 1 µl of a random hexamer (0.1 mg/ml), 4 min at 65 °C. The RT reaction mixture was added including pairs of primers, which were incubated for 45 min at 42 °C. The following pairs of primers were used: Basic FGF: F, 5′-AAT TAC AAC TCC AAG CAG AAG AGA GA-3′; R, 5′-TTA AGA TCA GCT CTT AGC AGA CAT-3′; HGF: F, 5′-CCA TGA ATT TGA CCT CTA TG-3′; R, 5′-ACT GAG GAA TGC CAC ACA-3′; IGF-1: F, 5′-AAG GAG GAA GGA TTC TCA TCT G-3′; R, 5′-TAT TTA GTC GCC TTT CCA GGT C-3′; PDGF-A: F, 5′-GCC CCT GCC CAT TCG GAA GAA GA-3′; R, 5′-GGC CAC CCT GAC GCC GTG GTG C-3′; IL-6: F, 5′-TTC CTC TCT GCT GCA AGA GAC T-3′; R, 5′-TGG ATC TCT CTG AAG GAC T-3′; LIF: F, 5′-CCC ATG AGG TTC TTG GC-3′;
R, 5′-CTC CTC TCT AGA AGG CCT G-3′; GAPDH: F, 5′-ACA TCA TCC CTG CAT CC-3′; R, 5′-CTC CTT GGA GGC CAT GT-3′.

**RESULTS**

*TNFα Induces Two Phases of JNK Activity Necessary to Inhibit Myoblast Differentiation*—TNFα was added to C2 cells during early differentiation stages, and JNK activity was measured by analyzing the phosphorylation state of c-Jun (Ser-63,73) (Fig. 1A). Two phases of c-Jun phosphorylation followed the addition of TNFα were observed; an immediate phase at 30–120 min and a late phase at 24 h (Fig. 1A). Similar results were obtained with an antibody to phospho-JNK, indicating that JNK is involved in c-Jun phosphorylation following TNFα treatment (Fig. 1A). Next, the expression of myogenic markers was analyzed (Fig. 1B). Addition of TNFα to DM reduced late expression of myogenin and myosin heavy chain (MyHC) (24–48 h in DM). Because TNFα inhibits the expression of myogenic markers and myoblast differentiation (16), we asked whether JNK was involved in the process. TNFα was added to C2 cells during early differentiation stages, and differentiation was assessed 48 h later by analyzing myotubes formation (Fig. 1C). Differentiation was significantly prevented when TNFα was included in the medium. The addition of a pharmacological inhibitor of JNK, SP600125, at a concentration preventing phosphorylation of c-Jun (Fig. 1C, lower panel) allowed the formation of myotubes in the presence of TNFα (Fig. 1C, upper panel). Partial rescue of differentiation in the presence of JNK inhibitor was also suggested by the expression of the early differentiation marker, myogenin, and the late marker, MyHC (Fig. 1C). These results suggest that JNK is involved in TNFα-mediated inhibition of myoblast differentiation.

*JNK1 but Not JNK2 Is Involved in Mediating the Effects of TNFα on Myoblasts*—To further investigate the inhibitory role of JNK in myogenesis downstream of TNFα and to identify the JNK isoforms involved, myogenic lines were derived from JNK1- and JNK2-null fibroblasts (17). Expression vectors encoding MyoD:estrogen receptor chimera protein were stably integrated into wild-type, JNK1-, and JNK2-null fibroblasts (17). Expression vectors encoding MyoD:estrogen receptor chimera protein were stably integrated into wild-type, JNK1-, and JNK2-null fibroblasts (17).
TNFα Inhibits Myoblast Differentiation via JNK1

Dependent on JNK1 and c-Jun Activities

A.

WT       JNK1-/-       JNK2-/-

48h DM    48h DM +TNFα

B.

Time (after TNF):

|            | 24h | 48h | 24h | 48h | 24h | 48h |
|------------|-----|-----|-----|-----|-----|-----|
| TNFα:      | -   | +   | -   | +   | -   | +   |
| Myogenin   | -   | +   | -   | +   | -   | +   |
| MyHC       | -   | +   | -   | +   | -   | +   |
| Tubulin    | -   | +   | -   | +   | -   | +   |

C.

Time (after TNF): 0 30min 2h 6h 24h 48h

TNFα:

| pc-Jun | c-Jun |
|--------|-------|
| WT     | WT    |
| JNK1-/-| JNK1-/-|
| JNK2-/-| JNK2-/-|

D.

Time (after TNF): 24h 48h

c-Jun-/-

| Myogenin | MyHC | Tubulin |
|----------|------|---------|
| WT       | WT   | WT      |
| JNK1-/-  | JNK1-/- | JNK1-/- |
| JNK2-/-  | JNK2-/- | JNK2-/- |

FIGURE 2. JNK1, but not JNK2, is involved in TNFα-mediated inhibition of myoblast differentiation. A, fibroblasts originated from JNK1 and JNK2 knock-out and wild-type mice expressing the MyoD:ER chimera protein were differentiated in DM and β-estradiol in the absence or presence of TNFα (20 ng/ml). Forty-eight hours following TNFα treatment, cells were immunostained with primary anti-MyHC antibody. DAPI staining was used to detect cell nuclei. B, the above cell lines were grown in DM in the absence or presence of TNFα for the indicated time periods, and proteins were extracted and analyzed by Western blotting using antibodies to myogenin (F5D) and MyHC (MF20). Tubulin was used as a loading control. C, the above cell lines were grown in DM in the absence of presence of TNFα for the indicated time periods, and proteins were extracted and analyzed by Western blotting using antibodies to phosphorylated c-Jun (pc-Jun) and to total c-Jun protein (c-Jun). D, fibroblasts originated from c-Jun knock-out mouse and expressing the MyoD:ER chimera protein were differentiated in DM and β-estradiol in the absence or presence of TNFα (20 ng/ml). Proteins were extracted at different time periods after TNFα treatment and analyzed by Western blotting using antibodies to myogenin (F5D) and MyHC (MF20). Tubulin was used as a loading control.

Entiation rate (not shown). Interestingly, TNFα significantly inhibited differentiation of wild type as well as of JNK2-null cells, but did not affect JNK1-null cells (Fig. 2A, lower panel). The expression of myogenin and MyHC proteins analyzed by Western blotting indicated the same; the levels of these proteins were not affected following TNFα treatment in JNK1-null cells, whereas they were significantly reduced in wild-type and JNK2-null cells (Fig. 2B). Interestingly, this effect of TNFα on the expression of myogenic markers was observed late (48 h in DM) rather than early (24 h in DM) (Fig. 2B). Similarly, C2 myoblasts expressing shRNA directed to JNK1 partially restored MyHC expression to TNFα-treated cells (supplemental Fig. S2). The results indicate that JNK1 but not JNK2 is involved in inhibition of differentiation by TNFα. Next, we examined JNK kinase activity toward c-Jun following TNFα treatment of the three cell lines. Addition of TNFα to the wild-type cell line (3T3 MyoD:ER) induced the same levels of myogenin and MyHC proteins, but did not reduce but rather induced expression of differentiation markers relative to control cells (Fig. 2D). Therefore, TNFα may have stimulated differentiation of this cell line in contrast to its inhibitory effect on wild-type and JNK2-null cells. Overall, TNFα did not inhibit differentiation of JNK1 and c-Jun-deficient myoblasts, indicating that these proteins mediate inhibition of differentiation by TNFα.

TNFα Induces a Wave of Myoblast Proliferation That Is Dependent on JNK1 and c-Jun Activities—The resistance of JNK1- and c-Jun-null cells to the inhibition of myoblast differentiation by TNFα was further investigated. JNK1- and c-Jun-null fibroblasts share certain defects in cell cycle, significantly reducing their proliferation rates (17, 18). Therefore, it is possible that TNFα induces proliferation of wild-type myoblasts and not of JNK1- and c-Jun-deficient myoblasts in a way that would explain its distinct effects on differentiation. We analyzed the percentage of C2 cells in S phase by BrdU incorporation (Fig. 3A). Twelve percent of C2 cells were in S phase following 24 h of growth in differentiation medium (DM; see “Experimental Procedures”). The number of C2 myoblasts in S phase was increased to 37% following treatment with TNFα.
**A.**

-SP

+SP

24h in DM

**B.**

3T3 MyoD:ER

WT

JNK2-/

JNK1-/

c-Jun-/

**C.**

|        | DM (24h) | DM+TNFα (24h) |
|--------|----------|---------------|
|        | G₀/G₁ | S | G₂/M | G₀/G₁ | S | G₂/M |
| WT     | 46.2  | 2.8 | 51   | 32    | 24 | 44   |
| JNK2-/-| 84.5  | 10.3| 5.2 | 63.5  | 25 | 11.5 |
| JNK1-/-| 90.5  | 6.6 | 2.9 | 90.7  | 5.2 | 4.1  |
| c-Jun-/-| 86.3 | 6.8 | 6.9 | 86    | 8.6 | 5.4 |

**D.**

pc-Jun    p-His H3    merge
The percentage of cells in S phase was decreased to 12% if the JNK inhibitor SP600125 was added to TNFα-treated myoblasts (Fig. 3A). Hence, TNFα induced proliferation of differentiating C2 cells, which was likely mediated by JNK. We further analyzed the percentage of cells in S phase following the addition of TNFα to different MyoD:ER cell lines (Fig. 3B). The addition of TNFα to wild-type and JNK2-null cells significantly increased the number of cells in S phase after 24 h in DM relative to cells not treated with TNFα (Fig. 3B, upper panel). In contrast, TNFα did not increase the percentage of JNK1- or c-Jun-null cells in S phase after 24 h in DM (Fig. 3B, lower panel). FACS analysis of the different cell lines further substantiated the notion that TNFα did not induce proliferation of JNK1- and c-Jun-null cells, whereas it induced significant proliferation of wild-type and JNK2-null cells (Fig. 3C). This is suggested by the proportion of cells in S and G1/M stages relative to cells in G0 stage of the cell cycle (Fig. 3C, left panel). In conclusion, TNFα induces the proliferation of myoblasts in a JNK1 and c-Jun-dependent manner.

The apparent correlation between proliferating myoblasts and c-Jun phosphorylation occurring 24 h following TNFα treatment may indicate that phosphorylated c-Jun was associated with proliferating cells. To further investigate this possibility, wild-type MyoD:ER myoblasts were grown for 24 h following their treatment with TNFα and were immunostained using anti-phospho-c-Jun, and anti-phospho-histone H3 (Fig. 3D). Phosphorylated histone H3 marks cells in mitosis. Interestingly, phospho-c-Jun staining was observed only in cells expressing phospho-histone H3 (Fig. 3D). Therefore, the second peak of c-Jun phosphorylation following TNFα treatment likely represents mitotic myoblasts. Interestingly, phospho-c-Jun appears to be excluded from DNA and is concentrated in what appears as centromeres of mitotic cells.

**TNFα Induces Myoblast Differentiation Indirectly—** The overt inhibition of differentiation by TNFα (see Figs. 1 and 3) could not be explained merely by the proliferating myoblasts, because not all myoblasts entered the cell cycle (see Fig. 3). Therefore, we hypothesized that the myoblasts that reentered the cell cycle could prevent differentiation of the remaining population of resting myoblasts. To investigate this possibility, we added Ara-C to differentiating cells treated with TNFα. Ara-C is an analogue of cytosine used in chemotherapy, which upon incorporation to newly synthesized DNA kills cells in the S phase. The addition of TNFα to differentiation medium almost abolished the formation of myotubes (Fig. 4A, left panel). However, when proliferating cells were eliminated by Ara-C treatment, most of the remaining cells formed myotubes despite the presence of TNFα. Similarly, Ara-C treatment restored the expression of myogenic markers like myogenin and MyHC inhibited by addition of TNFα to differentiating myoblasts (Fig. 4A, right panel). A different approach was used to arrest cell cycle at the G1 stage; thymidine was added to medium of myoblasts treated with TNFα. Thymidine block allowed for myoblast differentiation in the presence of a TNFα concentration normally preventing differentiation (supplemental Fig. S3). The above results suggest that cell cycle arrest or removal of proliferating cells prevents the inhibitory effect of TNFα, indicating that TNFα may not interfere directly with the differentiation of non-replicating myocytes. Consequently, we speculated that cells forced into the cell cycle by TNFα secrete growth factors/cytokines into the culture medium preventing the differentiation of the remaining myoblasts. To further investigate this possibility, we analyzed the differentiation of JNK1-/− myoblasts grown in conditioned medium isolated from either JNK1-/−, JNK2-/−, or wild-type myoblasts grown in the absence or presence of TNFα (Fig. 4B). Conditioned medium from JNK1-/− cells grown without or with TNFα did not affect formation of myotubes (left panel) and muscle gene expression (right panel) of JNK1-/− cells. However, conditioned medium originated from JNK2-/− or wild-type cells treated with TNFα completely abolished muscle differentiation of JNK1-/− cells. These results clearly indicate that wild-type and JNK2-/− cells treated with TNFα, but not JNK1-/− cells secreted factor(s) into the growth medium that prevented the differentiation of JNK1-/− cells.

**Myoblasts Express the Proinflammatory Cytokines IL-6 and LIF in Response to TNFα—** Assuming that TNFα induced the secretion of growth factors or cytokines into the medium that inhibited the differentiation of myoblasts, we screened for the expression of certain growth factors and cytokines. The selected factors all involved cell proliferation, stemness, and antagonism of differentiation. C2 cells were treated with TNFα, and 24 h later the expression of certain growth factors and cytokines was analyzed by semi-quantitative RT-PCR (Fig. 5A). The expression of some growth factors, known to inhibit myoblast differentiation, like bFGF, HGF, and PDGF was not affected by TNFα treatment. The expression of IGF-1, functioning both on proliferating as well as differentiating myoblasts, was down-regulated following TNFα treatment (Fig. 5A). The expression of two cytokines, IL-6 and LIF was substantially induced following TNFα treatment. We further analyzed the expression profile of these two cytokines in C2 cells treated with TNFα (Fig. 5B). Transcripts of IL-6 and LIF were identified in two peaks following the addition of TNFα to C2 cells; the first around 30 min to 2 h and the second around 24 h. This pattern is similar though not identical to that of c-Jun phosphorylation

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**FIGURE 3. TNFα induces proliferation of myoblasts that is dependent on JNK1 and c-Jun activities.** A, C2 myoblasts growing in DM for 2 h were treated with TNFα (20 ng/ml) and SP600125 (20 μM). To identify the nuclei involved in DNA synthesis, BrdU was added to DM 3 h before cells were fixed and immunostained, as described under “Experimental Procedures.” The total number of nuclei was identified by DAPI staining. Histogram represents the average of five different fields used to calculate the percentage of cells in the S phase. The percentage of cells in the S phase was determined by dividing the number of BrdU-stained nuclei by the number of DAPI-stained nuclei in each microscopic field. B, similar experiment as in A was performed with the different 3T3 MyoD:ER cell lines (wild type, JNK1−/−, JNK2−/−, and c-Jun−/−). The percentage of cells in S phase was determined on cells growing in GM, 6 and 24 h in DM. Histograms represent the average of five different fields used to calculate the percentage of cells in the S phase. C, propidium iodide FACS analysis of the same cell lines as in B. Cells were grown in DM for 2 h with treatment with TNFα, and collected for FACS analysis 24 h later. The experiment was performed twice with similar results. A typical analysis is presented in the right panel, and the percentage of cells at the different stages of the cell cycle (G0/G1, S, G2/M) in the left panel. D, wild-type 3T3 MyoD:ER cells grown in DM were immunostained 24 h after the addition of TNFα. Cells were stained with antibodies to phospho-c-Jun and to phospho-histone H3. Left panel, a typical field of cells representing few cells undergoing mitosis. Right panel, enlargement of selected mitotic cells.
following the addition of TNFα to C2 cells (see Fig. 1A), suggesting that IL-6 and LIF expression may be mediated by JNK activity. The involvement of JNK1 in the expression of IL-6 and LIF was investigated using the chimera MyoD:ER-expressing cell lines. Addition of TNFα to wild-type and to JNK2−/− myoblasts induced two phases of IL-6 and LIF expression, after 2 and after 24 h (Fig. 5C). In contrast, TNFα induced sustained expression of IL-6, but no expression of LIF in JNK1−/− myoblasts (Fig. 5C). This result indicates that LIF but not IL-6 expression is dependent on JNK1 activity.

Because TNFα stimulates NFκB activity, it was important to find out whether NFκB was also involved in the expression of LIF. For analyzing NFκB activation following TNFα treatments in the different cell lines, the cellular localization of p65, part of the NFκB complex, was investigated (supplemental Fig. S4). P65 was translocated from the cytoplasm to cell nuclei following the addition of TNFα to wild-type MyoD:ER myoblasts, indicating that NFκB was activated. Likewise, TNFα also induced the translocation of p65 into the nuclei of the other cell lines including JNK1-null myoblasts (supplemental Fig. S4). Therefore, it appears that NFκB activity is induced by TNFα in JNK1−/− myoblasts, which is not sufficient to induce the expression of LIF (see Fig. 5C). In another experiment, a myogenic cell line expressing MyoD:ER was derived from fibroblast cells originated from IKKβ-null mouse and therefore devoid of NFκB activity (19). Massive apoptosis of these myoblasts occurred 48 h after the addition of TNFα to these cells (not shown). Still, TNFα induced a constitutive expression of LIF in the absence of NFκB activity (supplemental Fig. S5A). The constitutive expression of LIF correlated well with constitutive activity of JNK following TNFα treatment (supplemental Fig. S5B). Therefore, TNFα-induced expression of LIF correlates with JNK activity and not with NFκB activity.

**TNFα-induced Inhibition of Myoblast Differentiation Is Mediated by LIF**—The above results suggest that LIF may be the secreted factor mediating the inhibitory effect of TNFα on myoblast differentiation. To further explore this possibility, recombinant LIF protein was added to the medium of JNK1−/− MyoD:ER cells. This cell line was selected because its differentiation process was not affected by TNFα, and it did not synthesize LIF in response to TNFα (see Figs. 2, 3, and 5). Addition of LIF largely inhibited the differentiation of JNK1−/− myoblasts judged by the expression of myogenin and MyHC in Western analysis and by immunostaining of myotubes with an antibody to MyHC (Fig. 6A). This inhibition is not a consequence of cell proliferation, because LIF did not increase the percentage of JNK1−/− cells in S phase (data not shown). Therefore, the negative effect of LIF on JNK1−/− MyoD:ER differentiation is reminiscent of conditioned medium originating from wild-type and JNK2−/− cells treated with TNFα (see Fig. 4B). Consequently, our assumption is that LIF is the cytokine secreted by myoblasts in response to TNFα, and it inhibits the ability of resting myoblasts to differentiate. To test this possibility, a LIF-neutralizing antibody was added to C2 myoblasts grown in DM in the absence or presence of TNFα (Fig. 6B). As observed before, TNFα abolished the differentiation of myoblasts. The neutralizing antibodies added to the differentiation medium (DM) restored differentiation of myoblasts, whereas control antibody did not reverse the effect of TNFα (Fig. 6B). Despite the partially restored differentiation, the inclusion of LIF-neutralizing antibody did not prevent proliferation of myoblasts by TNFα (BrdU staining, Fig. 6C). Interestingly, addition of recombinant LIF to C2 myoblasts at a concentration pre-
**DISCUSSION**

**TNFα affects many levels of muscle development and maintenance.** Our study deals with the stages of myoblast growth and differentiation. The role of TNFα has been extensively studied because it is implicated both in the build up of muscle under normal healthy conditions and its degeneration during many chronic diseases. Over the years in *vitro* studies have demonstrated that at high concentrations, TNFα induces myoblast proliferation, while low concentrations induce differentiation (20–22). The opposite effects are likely a consequence of inducing different intracellular signaling pathways (21, 22). Several mechanisms were suggested to describe inhibition of myoblast differentiation (11, 12, 23–25). A few studies indicated the role of JNK in the inhibitory function of TNFα on myogenesis (13, 26). These studies showed that JNK was involved in inhibiting IGF-1 signaling, known to promote muscle growth and differentiation. Yet, none of these studies asked whether JNK was involved in myoblast proliferation, differentiation, or both. The results of the present study indicate two distinct activities of TNFα mediated by the JNK pathway, a pro-proliferative and an anti-differentiative pathway. Of note, all the experiments of this study were performed with cell lines, either fibroblasts expressing MyoD:ER chimera, or C2 myoblasts. Therefore, the conclusions drawn are limited to cell lines, and further studies with primary myoblasts should support more general conclusions.

**TNFα Induces Proliferation of Myoblast through the JNK Pathway—**Our experiments indicate that JNK activity is induced following treatment of myoblasts with TNFα. JNK activity is apparent by a typical two-peak phosphorylation of c-Jun at Ser-63 and -73; the first after 30 min (immediate) and the second after 24 h (delayed). TNFα does not affect the initiation of myogenin expression (6–12 h), but gradually reduces its later expression (24–48 h). This pattern of myogenin expression is well correlated with the TNFα-induced entry of myoblasts into the cell cycle with maximum cells in the S phase after 24 h. Proliferation is completely prevented if a pharmacological inhibitor of JNK is added to cells, preventing their differentiation (not shown) did not induce their proliferation (Fig. 6C). In summary, we suggest a bimodal TNFα activity: it directly promotes myoblast proliferation through activation of JNK1, and it prevents the differentiation of non-dividing myoblasts through its induced secretion of LIF by the proliferating myoblasts.

**FIGURE 5.** TNFα induces the expression of the proinflammatory cytokines IL-6 and LIF in myoblasts. A, C2 myoblasts growing in DM were treated with TNFα, and 24 h later, RNA was extracted and semi-quantitative RT-PCR analysis was performed using pairs of primers to different growth factors and cytokines as described. B, C2 myoblasts were grown as in A, and RNA was extracted at different time points following the addition of TNFα, as indicated. Semi-quantitative RT-PCR analysis was performed using pairs of primers complementary to IL-6, LIF, and GAPDH. C, different 3T3 MyoD:ER cell lines were grown in differentiation medium and β-estradiol in the absence or presence of TNFα. RNA was extracted at the indicated time points and semi-quantitative RT-PCR was performed using pairs of primers complementary to IL-6, LIF, and GAPDH.
defective myoblasts, these cells may respond to TNFα by sustained induction of p38, promoting cell cycle arrest and subsequent differentiation (29–32). This model awaits further investigation.

An interesting finding of the present study is the simultaneous phosphorylation of c-Jun and the wave of proliferating myoblasts occurring 24 h following the addition of TNFα. The involvement of the JNK pathway and c-Jun in cell proliferation is well known, though the exact roles in cell cycle progression are poorly defined. c-Jun was suggested before to be involved in G1 progression via the expression of cyclin D1 (33). However, c-Jun may also be involved in mitosis. Nocodazole-released cells progressing through mitosis exhibit high phosphorylation of c-Jun at Ser-63 that disappears by the end of mitosis (33).

Moreover, recent studies indicated that JNK is associated with the centrosome and is active from S phase through anaphase (34) and that JNK activity is necessary to complete mitosis in myoblasts (35; Figure 6). Here, we find that following treatment of myoblasts with TNFα, the expression of c-Jun is upregulated and may exhibit separate activities on myoblasts. TNFα induced the expression of IL-6 but not of LIF in JNK1-deficient myoblast cells, indicating that JNK1 affects the expression of LIF in myoblasts. Therefore, a non-autonomous effect mediated by secreted factor(s) was considered. It was demonstrated before that incubation of differentiated C2C12 cells with TNFα induced the expression of proinflammatory cytokines like IL-6 and IFN-γ (37). In this study we find that TNFα induces the expression of cytokines of the same family, IL-6 and LIF. Although, these two cytokines appear functionally redundant in many biological systems, our results indicate that they are differently regulated by TNFα and may exhibit separate activities on myoblasts. TNFα induced the expression of IL-6 but not of LIF in JNK1-deficient myoblast cells, indicating that JNK1 affects the expression of LIF in myoblasts. Because TNFα also induces the activity of NFκB, which by itself is known to regulate the expression of many cytokines, we investigated the involvement of NFκB in the expression of these cytokines. We took advantage of a myo-
blasts cell line devoid of the I KKβ gene and thus exhibiting no NFκB activity (19). In response to TNFα, these cells expressed constitutive levels of LIF found in correlation with constitutive phosphorylation of c-Jun. Therefore, the expression of LIF is dependent on JNK and not on NFκB activity. The effect of IL-6 was not further analyzed in the present study, because its expression in JNK1−/− myoblasts treated with TNFα did not inhibit their differentiation. IL-6 was suggested as a myoblast differentiation-promoting cytokine in previous studies (38, 39). Therefore, IL-6 is most likely not the cytokine mediating the inhibitory effects of TNFα on myoblast differentiation.

LIF is expressed following muscle injury and is necessary for muscle regeneration (40, 41). LIF was shown to promote proliferation and inhibit differentiation of C2C12 and primary myoblasts (42, 43). Signaling molecules like Stat3 (44) and ERK MAPK (45) function downstream of LIF in promoting proliferation of myoblasts. Interestingly, none of the previous studies distinguished the positive effect of LIF on proliferation and inhibit differentiation of C2C12 and primary myoblast cell line devoid of the IKKα/β gene. Therefore, LIF is most likely not the cytokine mediating the inhibitory effects of TNFα on myoblast differentiation.

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