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Tumor Necrosis Factor Alpha Induces LIF Expression Through ERK1/2 Activation in Mammary Epithelial Cells

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ABSTRACT

It has been reported that expression of tumor necrosis factor superfamily members occur at the onset of the mammary gland post-lactational involution. One of these proteins, tumor necrosis factor alpha (TNFα), is a major mediator of inflammation that is able to induce expression of several cytokines. Leukemia inhibitory factor (LIF) is an inflammatory cytokine that is induced and plays a fundamental role during post-lactational involution of the mammary gland. Therefore, our goal was to determine whether TNFα activity in the mammary epithelium might include regulation of LIF expression. This biological role would increase the significance of TNFα expression at the end of lactation. Our results show that TNFα was able to induce LIF transcription through ERK1/2 activation in a non-tumorigenic mouse mammary epithelial cell line, SCp2. We found that activation of TNFα receptor-2 (TNFR2) was specifically involved in triggering this signaling pathway. In addition, our data suggest the participation of AP-1 transcription factor family members in this pathway. We determined that TNFα treatment induced c-fos transcription, and blocking AP-1 activity resulted in a significant inhibition of TNFα-induced LIF expression. Finally, we found that TNFα was also able to trigger LIF expression and ERK1/2 activation in the mouse mammary gland *in vivo*. Therefore, our data suggest that TNFα may contribute to mammary gland involution by, among other activities, eliciting LIF expression through ERK1/2 and AP1 activation. J. Cell. Biochem. 110: 857–865, 2010. © 2010 Wiley-Liss, Inc.

KEY WORDS: LIF; TNFα; AP-1; MAMMARY EPITHELIAL CELLS; MAMMARY GLAND; INVOLUTION

Post-lactational involution of the mammary gland is one of the most dramatic examples of developmentally regulated epithelial apoptosis in mammals. Initially, removal of the pups and the subsequent milk stasis cause the induction of local factors that lead to epithelium apoptosis [Strange et al., 1992; Li et al., 1997]. The relevance of the transcription factor STAT3 in post-lactational involution has been well demonstrated. This transcription factor is essential for timely initiation of post-lactational regression and orchestrates the processes of cell death and tissue remodeling that occur during the first 6 days of involution in the mouse [Chapman et al., 1999]. In addition, it has been shown that leukemia inhibitory factor (LIF), which is induced only a few hours after lactation interruption, is responsible for the activation of this transcription factor in mammary epithelial cells [Kritikou et al., 2003; Schere-Levy et al., 2003; Quaglino et al., 2007]. Induction of other factors such as the death receptor ligands, Tumor necrosis factor (TNF) superfamily as FASL (*Tnfsf6*), TRAIL (*Tnfsf10*), TWEAK (*Tnfsf12*) and TNFα itself, has also been observed within 12 h after weaning [Clarkson et al., 2004; Baxter et al., 2006].

TNFα is a multifunctional cytokine produced by many cell types in response to inflammation, infection, and environmental stress [Urban et al., 1986; Larrick and Wright, 1990]. The cellular effects of TNFα are initiated by two distinct surface receptors: TNFR1 (p55) and TNFR2 (p75), which are expressed in most cell types. Although

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these two receptor forms share some sequence identity in their extracellular domains, their intracellular regions are quite different. Therefore, upon interaction with their ligands, each receptor may activate different signal transduction pathways that, in turn, would generate specific cell responses [Lewis et al., 1991]. For example, in epithelial cells, TNFα-induced epiderma1 growth factor receptor expression is mediated by TNFR1, while a positive feedback on TNFα transcription occurs through TNFR2 activation [Kalthoff et al., 1993; Tartaglia et al., 1991, 1993]. In addition, it has been reported that the specific augmentation of TNFR2 in breast cancer cells might be relevant for cancer promotion, mediating TNFα effect on cell proliferation and survival [Garcia-Tunon et al., 2006]. Although the specific pathways triggered by each receptor are still poorly understood [Kyriakis and Avruch, 2001], the intracellular cascades would converge on key transcription factors such as NFκB and AP-1, allowing signaling integration at the level of gene expression regulation [Baud and Karin, 2001].

It has been shown that TNFα is able to activate ERK1/2 and AP-1 in tissues different from the mammary gland [Theiss et al., 2005; Srivastava et al., 2007]. In addition, ERK1/2, AP-1 and c-Fos activation have been observed in mammary cells after weaning [Jaggi et al., 1996; Marti et al., 1999; Zhao et al., 2002]. Here, in an attempt to integrate different factors and signaling cascades involved in mammary gland involution, we analyze the ability of TNFα to regulate LIF expression in mammary epithelium. Our results show that TNFα is able to induce LIF expression through ERK1/2 activation in mammary epithelial cells in culture and in vivo.

MATERIALS AND METHODS

CELL CULTURE AND TREATMENTS

Non-tumorigenic mammary epithelial cells SCp2 were routinely maintained as indicated previously [Blaustein et al., 2004]. Approximately 1–2 × 10^5 SCp2 cells were plated into culture plates with Dulbecco's modified Eagle's medium: F-12 (DMEM: F-12; Invitrogen) supplemented with 2% fetal bovine serum, insulin (5 μg/ml, Sigma) and gentamicin (50 μg/ml, Invitrogen). For TNFα treatment (20 ng/ml, Sigma), this medium was replaced by serum-free DMEM/F-12, and cells were treated for the indicated time or left untreated (controls). MAPKs inhibitors, PD98059 50 μM and SP600125 25 μM, were added to the cells 1 h before treatments and were left together with TNFα during the indicated times. Mouse polyclonal blocking antibodies against TNFR1 (p65) and TNFR2 (p75) were added 30 min before treatments as previously described [Rivas et al., 2008].

ANIMALS

Balb/c mice from the University of Buenos Aires mouse colony were maintained in a pathogen-free temperature-controlled environment on a 12 h/12 h light/dark cycle and given sterilized laboratory chow and water ad libitum in accordance with the NIH Guide for the Care and Use of Laboratory Animals. Mammary glands were aseptically removed from the mice at lactation phase (8 days after delivery); and 6 h, 20 and 48 h after weaning (pups were removed after 5–7 days of nursing). Mammary glands were then frozen in liquid nitrogen for RNA extraction or immersed in 10% buffered formalin and then embedded in paraffin for histological studies. To investigate the effect of TNFα on lactating mammary glands, four mice that had been nursing their pups for 7–10 days, were inoculated subcutaneously with 50 μl of saline solution either containing (left #4 fat pads) or not (right #4 fat pads) 1 μg of TNFα. Mammary glands were either removed or frozen in liquid nitrogen for RNA extraction 6 h after inoculation, or immersed in 10% buffered formalin and then embedded in paraffin for histological studies 24 h after treatment.

RNA ISOLATION AND mRNA QUANTIFICATION

RNA was prepared using the TRIZOL reagent (Invitrogen) according to the manufacturer's instructions. For reverse transcription-PCR (RT-PCR) analysis, cDNA was generated from 2 μg of total RNA using MMLV reverse transcriptase (Promega). Quantitative Real-Time PCR data were acquired and analyzed using the Opticon Monitor System (MJ Research Inc.). All samples were analyzed for actin expression in parallel within the same run. For each sample, the amplification plot and the corresponding dissociation curves were examined. Experiments were always run in triplicate and repeated at least twice. Bars indicate means ± SE from at least three independent experiments.

WESTERN BLOT ANALYSIS

Cells were lysed in 180 μl RIPA protein extraction buffer [50 mM Tris–HCl, 150 mM NaCl, 1% triton, 0.25% sodium deoxycholate, 1 mM EDTA pH 7.4] supplemented with proteases (Protease inhibitor cocktail set I, Calbiochem) and phosphatase inhibitors (1 mM NaF, 1 mM Na_2VO_4). Samples were homogenized and further disrupted by passage through a 21-gauge needle (8–10 times). They were subsequently incubated on ice for 30 min and centrifuged at 9,500*g* for 20 min at 4°C. Supernatants were transferred to a fresh tube and protein concentration was determined by Bradford's method. Cleared lysates were combined with SDS sample buffer (50 mM Tris pH 6.8, 2% SDS, 0.1% bromophenol blue, 10% glycerol, 100 mM DTT), boiled for 8 min and resolved by SDS-PAGE. Western blot analysis were carried out as described previously [Schere-Levy et al., 2003]. Membranes were incubated ON at 4°C with the appropriate dilution of the following primary antibodies: anti pERK (SC-7383), anti pP38 (SC-7973), anti pAKT (SC-16646-R) or anti pJNK (SC-6254) followed by incubation with horseradish peroxidase-conjugated anti-mouse, rabbit or goat secondary antibodies (all antibodies from Santa Cruz Biotechnology). Immunoreactive protein bands were visualized by enhanced chemiluminescence detection (ECL - Plus System, Amersham Biosciences).

AP-1 DECOY CDODN TRANSFECTION

SCp2 cells were plated in complete media and allowed to grow overnight to 70–80% confluence in 6-well plates. Cells were transfected using Lipofectamine Plus Reagent (Invitrogen), according to the protocol indicated by the manufacturer. Design and construction of the AP-1 circular dumbbell decoy oligodeoxynucleotide (CDODN) and the control mismatched AP-1 (MODN) has been previously described [Ahn et al., 2002]. Briefly, CDODN comprises two loops and one stem containing two AP-1 consensus sequences in tandem: 5′ TACTGAGTCTTCTGCAAAAGCAGAA-GACTCAGTACTTAAG 3′ and for the circular dumbbell mismatched

AP-1 decoy (MODN) the sequence was 5′ TTTAGAGTCTTCTG-CAAAAGCAGAAGACTCTAAACTTAAG 3′. CDODN and MODN were annealed for 2 h with a temperature descent from 80 to 25°C. T4 DNA ligase (1 unit) was added to the mixture, followed by incubation for 24 h at 16°C to generate a covalently ligated dumbbell-shaped decoy ODN molecule. For transfection 50 μM of ligated CDODN or MODN were added to the cells. After transfection, cells were treated with or without TNFα for 16 h.

ELECTROPHORETIC MOBILITY SHIFT ASSAYS (EMSA)

Preparation of nuclear extracts and EMSA were performed as described previously [Tanos et al., 2005]. Nuclear proteins were extracted from cells culture on 10 cm plates, scraped into 1.0 ml of cold phosphate buffered saline (PBS); non-adherent cells were pelleted 10 s and resuspended in 400 μl cold buffer A (10 mM HEPES KOH pH 7.9 at 4°C, 1.5 mm $MgCl_2$, 10 mM KCl, 0.5 mM dithiothreitol, 0.2 mM PMSF) by flicking the tube. Cells were allowed to swell on ice for 10 min, and then vortexed for 10 s. Samples were centrifuged for 10 s and the supernatant fraction was discarded. The pellet was resuspended in 50 μl of cold buffer C (20 mM HEPES - KOH pH 7.9, 25% glycerol, 420 mM NaCl, 1.5 mM MgCl2, 0.2 mM EDTA, 0.5 mM dithiothreitol, 0.2 mM PMSF) and incubated on ice for 20 min for high salt-extraction. Insoluble cellular material was removed by centrifugation for 2 min at 4°C and the supernatant fraction (containing nuclear DNA binding proteins) was stored at −70°C. Two micrograms of protein were incubated at room temperature with 1 μg of poly(dI-dC) and 0.1 μg of salmon sperm DNA in 20 μl of binding buffer (12 mM HEPES, pH 7.8, 60 mM KCl, 2 mM $MgCl_2$, 0.12 mM EDTA, 0.3 mM DTT, 0.3 mM PMSF, 12% glycerol) for 15 min. Complementary synthetic oligonucleotides containing a canonical AP-1 site were obtained from Promega and labeled with [γ-^{32}P]ATP using T4 polynucleotide kinase (Invitrogen). Labeled oligonucleotides were purified using sephadex G-25 columns (Amersham Biosciences) and (20,000 cpm/reaction) added to the reactions for an additional 15 min. Complexes were analyzed on nondenaturing (4.5%) polyacrylamide gels in TGE buffer (40 mM Tris, 270 mM glycine, 2 mM EDTA, pH 8.0) run at 13 V/cm at 4°C. Unlabeled AP-1 was added to compete AP-1 binding to the labeled probe.

IMMUNOHISTOCHEMICAL STUDIES

Mammary glands were fixed in 10% buffered formalin and embedded in paraffin using standard procedures [Schere-Levy et al., 2003]. Briefly, after, paraffin sections were de-waxed, they were re-hydrated and used for immunohistochemical studies. Assays were carried out using a polyclonal LIF antibody (SC 1336) and a monoclonal pERK1/2 antibody (SC-7383) from Santa Cruz Biotechnology, Inc. Detections were performed using the Vectastain Elite ABC immunoperoxidase system (Vector Laboratories) following manufacturer's instructions with DAB (3′-3 diaminebenzidine) (DAKO, Denmark A/S) as chromogen. LIF and ERK1/2 immunostainings were qualitatively evaluated. Negative controls were performed replacing the primary antibodies with normal rabbit serum.

STATISTICAL ANALYSIS

Results are expressed as mean ± SD. The differences between control and experimental groups were analyzed by one-way ANOVA followed by Tukey post test. When only two groups were compared, Student's *t*-test was used (STATISTICA, Statsoft, Tulsa, OK).

RESULTS

TNFα INDUCES LIF EXPRESSION THROUGH ERK1/2 ACTIVATION

To determine whether TNFα could mediate LIF expression induction in the mammary gland, we analyzed the response of the mouse mammary epithelial cell line SCp2 to TNFα treatment. The results show that this factor caused a significant induction of LIF expression (Fig. 1A). We found that this effect was already detected 30 min after TNFα treatment and was maximal after 2 h. Afterwards, LIF mRNA

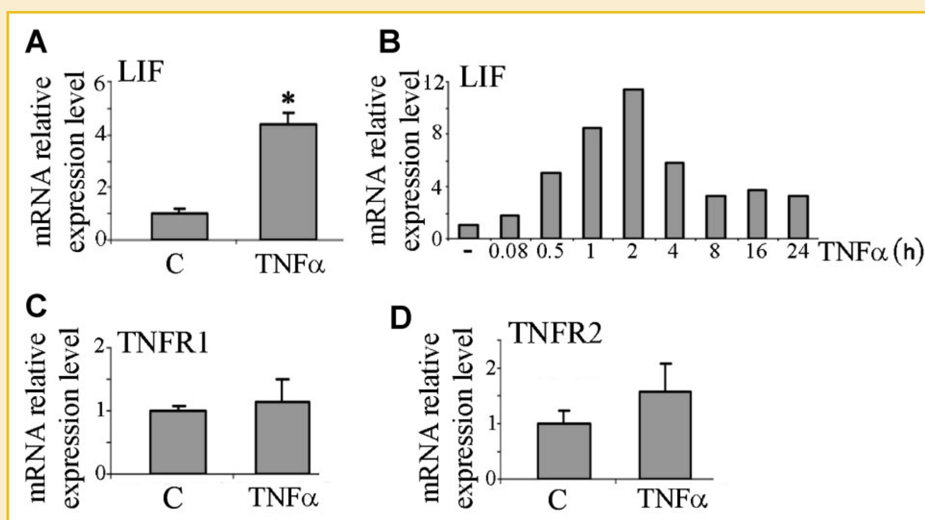

Fig. 1. Effect of TNFα treatment on LIF, TNFR1 and TNFR2 expression in SCp2 mammary epithelial cells. Expression levels of LIF (A,B) and TNFα receptors (C,D), relative to actin expression were determined by quantitative RT-PCR in SCp2 cells treated with TNFα 20 ng/ml for 16 h, TNF) and shown as fold induction compared to untreated controls. Bars indicate means ± SE from at least three independent experiments. Significant increase compared to control ($^*P < 0.0005$) determined by ANOVA test.

levels decreased; nevertheless, they were significantly higher than controls for up to 24 h (Fig. 1B). We have also analyzed the presence of TNFα receptors in SCp2 cells. We found that TNFR1 and TNFR2 were expressed in these cells, but their levels were not significantly affected by TNFα treatment (Fig. 1C,D).

In order to study the signaling cascades activated by TNFα treatment in SCp2 cells, we examined the phosphorylation levels of MAPKs and AKT. We found that p-ERK1/2 levels were increased and reached a peak 5 min after TNFα addition, while maximum levels of pJNK were detected 25 min later, when the intensity of p-ERK1/2 was already declining. On the other hand, TNFα treatment did not induce relevant changes of P38 or AKT phosphorylation levels in these cells (Fig. 2A).

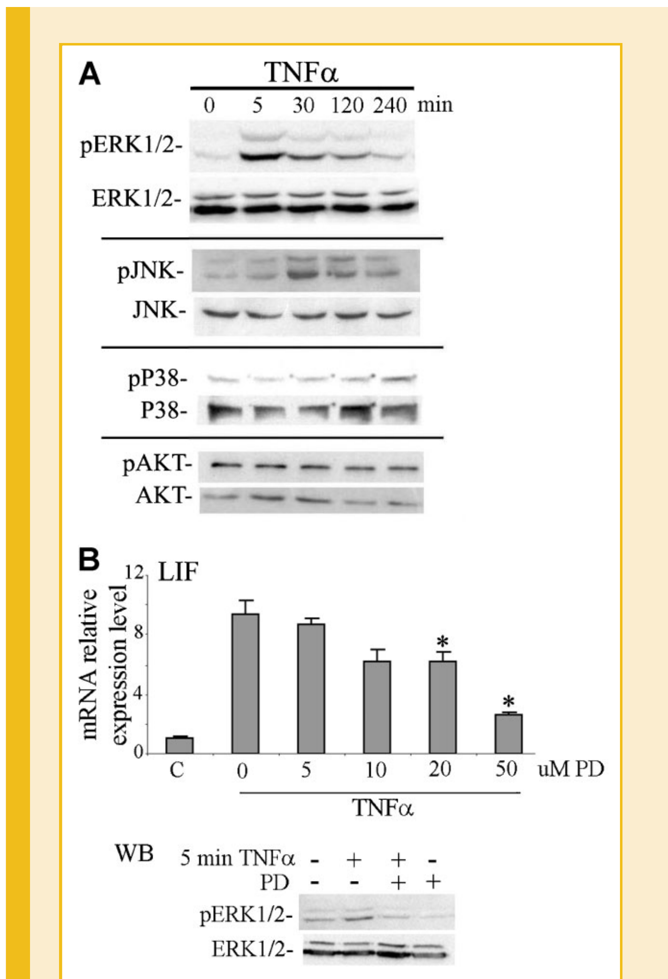

Fig. 2. Kinase activation patterns and LIF transcriptional levels in TNFα-treated, SCp2 mammary epithelial cells. Scp2 cells were treated with TNFα (20 ng/ml) for the indicated times and Western blot analysis of kinase phosphorylation levels were performed using phospho-specific antibodies against ERK1/2, JNK, p38, and AKT (upper panels). The same samples were blotted and challenged with antibodies to account for total kinase expression in each case (lower panels) (A). LIF expression, relative to actin, was analyzed by qRT-PCR, upon TNFα (20 ng/ml, 16 h) treatment in the presence of increasing doses of the MEK inhibitor PD98059 (PD) (B). Significant difference compared to TNFα treatment (0 μM PD) ($^{*}P < 0.0005$) determined by ANOVA test. The lower panel shows a representative Western blot analysis that illustrates the inhibitory activity of PD 50 μM on ERK1/2 phosphorylation.

To investigate the signal transduction pathways involved in TNFα-induced LIF expression, qRT-PCR assays were performed after pre-incubating cells with different pharmacological kinase inhibitors. Consistently with the results shown in Figure 2A, no significant alterations on LIF expression were observed when AKT or P38 pathway inhibitors were used (data not shown). On the other hand, the MEK-ERK1/2 inhibitor PD98059 (PD) reduced TNFα-induced LIF expression in a dose-dependent manner (Fig. 2B). Then, we analyzed the involvement of JNK in the regulation of LIF expression. Interestingly, we found that treatment of SCp2 cells with the JNK inhibitor SP 600125 (SP) resulted in a significant increase of LIF expression levels, induction that was strongly enhanced by TNFα addition (Fig. 3A). These observations suggested that JNK activation could play an inhibitory role on LIF expression regulation. In addition, we found that SP + TNFα co-treatment was able to prevent ERK1/2 de-phosphorylation, maintaining pERK1/2 high levels for up to 5 h (Fig. 3B). This effect is worthy of note since a single treatment of TNFα induced a transient peak of ERK1/2 that was already declining 30 min after addition (see Fig. 2A). Therefore, SP + TNFα-induced LIF over-expression might be mediated by ERK1/2 sustained activation. To test this hypothesis, SCp2 cells were treated with TNFα, ERK1/2 and JNK inhibitors (PD and SP, respectively). The results show that LIF over-induction caused by SP

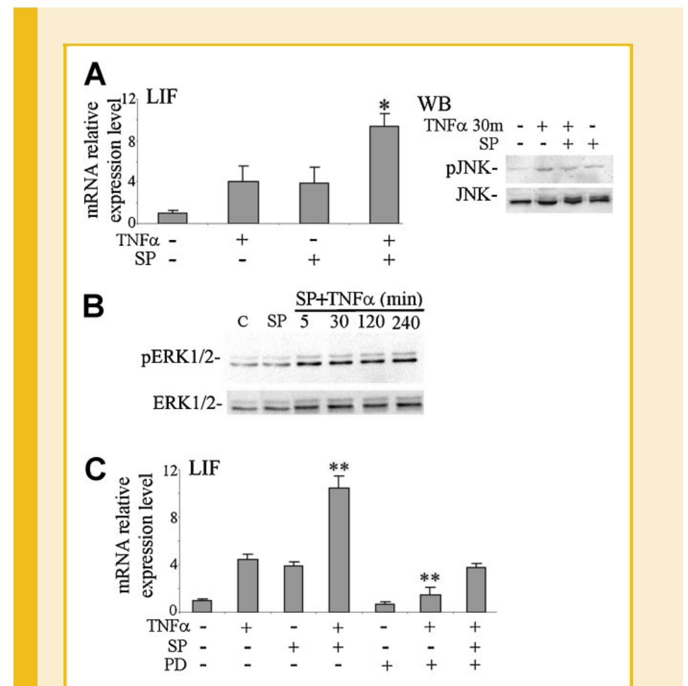

Fig. 3. Effect of inhibiting JNK activity on LIF expression and on ERK1/2 activation in SCp2 cells. qRT-PCR analysis of LIF expression, relative to actin, upon TNFα (20 ng/ml, 16 h) treatment in the presence or absence of the JNK inhibitor SP600125 (SP, 25 μM). On the right, the Western blot analysis shows SP (25 μM) inhibitory activity on JNK phosphorylation (A). Western blot analysis of total and phospho-ERK1/2 levels from cells pre-treated (30 min) with SP (25 μM) with or without TNFα for 5 min, 30 min, 2 h and 4 h (B). qRT-PCR analysis of LIF expression induction upon TNFα treatment (20 ng/ml, 16 h) in the presence, or absence, of either SP (25 μM) or PD (50 μM) or both (SP + PD) (C). Significant difference compared to treatment with TNFα without SP or PD ($^{*}P < 0.005$, $^{**}P < 0.0005$) determined by ANOVA test.

treatment was abolished by co-treatment with PD (Fig. 3C). These results suggest that ERK1/2 activation also mediates SP ability to enhance TNFα-induced LIF expression.

Although TNFα-induced rapid changes in LIF mRNA levels (Fig. 1B), the activity of pharmacological inhibitors on LIF expression was analyzed 16 h after treatment because we considered that the long-term effects were more significant for determining relevant signaling pathways in mammary gland physiology. However, this decision inhibits us to firmly establish that ERK1/2 activation directly modulates LIF transcription in the SCp2 cells.

TNFα INDUCES ERK1/2 ACTIVATION AND LIF EXPRESSION THROUGH TNFR2

Two types of high affinity TNFα receptors have been described: a 55-kDa receptor (TNFR55 or TNFR1) and a 75-kDa receptor (TNFR75 or TNFR2). In order to determine their involvement in TNFα-mediated LIF expression induction, we used specific blocking antibodies against each of these receptors. Thus, SCp2 cells were pre-incubated with anti-TNFR1 or TNFR2 mouse blocking antibodies prior to TNFα treatment. Interestingly, while only a partial reduction of TNFα-induced LIF expression was observed upon blocking TNFR1, abolishment of TNFα–TNFR2 binding resulted in abrogation of TNFα-mediated LIF expression induction (Fig. 4A). We then analyzed the effect of blocking each TNF-R on ERK1/2 phosphorylation. Figure 4B shows that both treatments down-regulate TNFα-induced ERK1/2 activation; although TNFR2 blockade resulted in a stronger inhibitory effect. These results suggest that signals originated through TNFR2 are necessary for triggering LIF expression induction.

Fig. 4. Role of TNFR1 and TNFR2 on TNFα-induced LIF expression in SCp2 cells. qRT-PCR analysis of LIF expression, relative to actin, upon TNFα treatment (20 ng/ml, 6 h) with or without pre-treatment (30 min) with 5 μg/ml blocking antibodies anti-TNFR1 (p55) or anti-TNFR2 (p75) or control isotype antibody (rabbit IgG). Significant difference compared to TNFα treatment without blocking antibodies (*$P < 0.0005$) determined by ANOVA test (A). Western blot analysis of phosphorylated (upper panel) and total (lower panel) ERK1/2 levels in cells pre-incubated (30 min) with blocking antibodies anti-TNFR1 or anti-TNFR2 or control isotype antibody (rabbit IgG) and then treated with TNFα (20 ng/ml, 5 min) (B).

LIF EXPRESSION REGULATION BY TNFα INVOLVES AP-1 ACTIVATION

It has been demonstrated that milk accumulation after weaning triggers AP-1 activation, which in turn results in the transcriptional induction of several target genes [Jaggi et al., 1996; Marti et al., 1999]. Analysis of LIF promoter sequences revealed the presence of two AP-1 sites located at positions −1866 and −3412. Therefore, we examined whether TNFα induced AP-1-DNA binding. Electro-mobility shift assays (EMSA) were performed on labeled AP-1 oligonucleotides that were pre-incubated with nuclear extracts from mammary epithelial cells treated or not with TNFα. We found a detectable AP1-DNA binding activity in SCp2 cells that reached a maximum 30 min after TNFα addition (Fig. 5A). To further investigate AP1 participation in TNFα-induced LIF transcription induction in mammary epithelium, an assay using an AP-1 circular dumbbell decoy oligodeoxynucleotide (CDODN) was performed. Then, SCp2 cells were either transfected with the AP-1 CDODN or an AP-1 mismatched mutated decoy oligodeoxynucleotide (MODN) before TNFα treatment. It was previously reported that this CDODN is specifically recognized by the AP-1 complex and showed an enhanced stability compared to lineal annealed AP-1 oligo decoy [Ahn et al., 2002]. By qRT-PCR we determined that mutated AP-1 MODN transfection did not alter TNFα ability to induced LIF expression. Conversely, TNFα treatment was unable to induce LIF expression in AP-1 CDODN transfected cells (Fig. 5B). These results show that titration of AP-1 transcription factors prevented TNFα-induced LIF expression. Furthermore, we investigated the effect of TNFα on the expression levels of *c-fos*, a major component of AP-1 transcription factor complexes. Interestingly, we found that TNFα treatment stimulated *c-fos* transcription (Fig. 5C) and that, similarly to LIF expression, this effect was blocked by a MEK inhibitor (PD) and enhanced by a JNK inhibitor (SP) (Fig. 5D). It is relevant to point out that c-Fos may participate in the formation of AP-1 complexes and therefore perform as an AP-1 component in a second wave of transcription induction involving multiple genes. Interestingly, we found that TNFα mediated induction of *c-fos* expression would be also mediated by ERK 1/2 and inhibited by JNK activation because pre-treatment with PD and SP exerted a similar effect to that observed on LIF expression regulation (Fig. 5D).

TNFα INDUCES LIF EXPRESSION AND ERK1/2 ACTIVATION IN THE MOUSE MAMMARY GLAND

TNFα and LIF gene expression were examined in lactating and involuting mouse mammary glands by qRT-PCR. Both genes showed very low expression during lactation and a steep increase after weaning (Fig. 6A,B). Similarly, TNFR1 and TNFR2 expression were induced during involution (Fig. 6C,D). In order to study TNFα ability to induce LIF expression in the mammary gland, we treated lactating glands with TNFα when endogenous LIF expression is at its lowest level. Interestingly, by qRT-PCR, we determined that TNFα-treated glands showed a significant increase in LIF expression

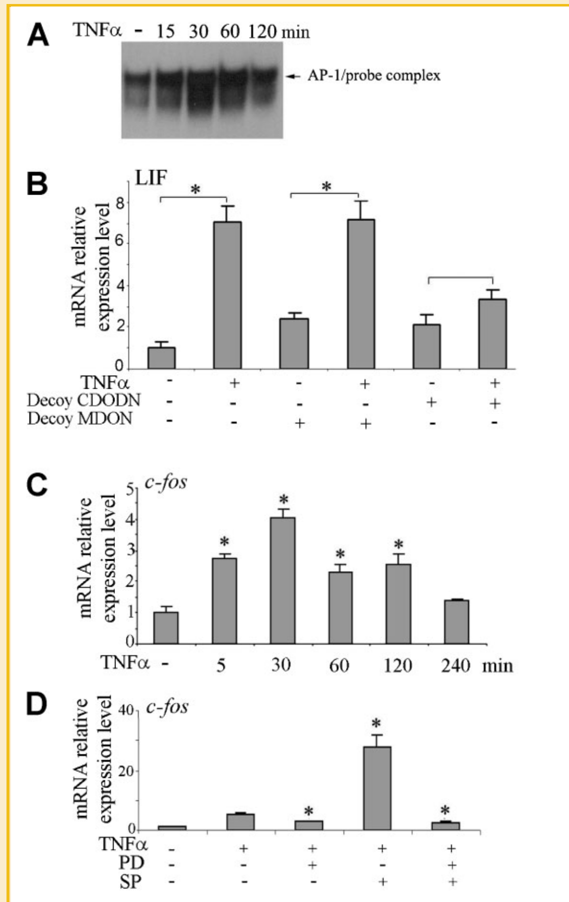

Fig. 5. AP-1 involvement in TNFα-induced LIF transcription. Electromobility shift assays (EMSA) of nuclear extracts from TNFα treated (20 ng/ml) and non-treated SCp2 cells at indicated times. Nuclear proteins were incubated with a labeled oligodeoxinucleotide encoding the AP-1 binding consensus sequence. The arrow shows the position of the AP-1-binding protein complex (A). qRT-PCR analysis of LIF expression, relative to actin, upon TNFα treatment (20 ng/ml, 16 h) of SCp2 cells that were previously transfected with either 100 nmol/L AP-1 oligo decoy CDODN, a mismatched AP-1 decoy MODN or not transfected (B). qRT-PCR analysis of *c-fos* expression, relative to actin, upon TNF (20 ng/ml) treatment during the indicated times (C) and in the presence or absence of either SP or PD or both (SP + PD) (D). Significant increase compared to the untreated control ($^*P < 0.0005$) determined by ANOVA test.

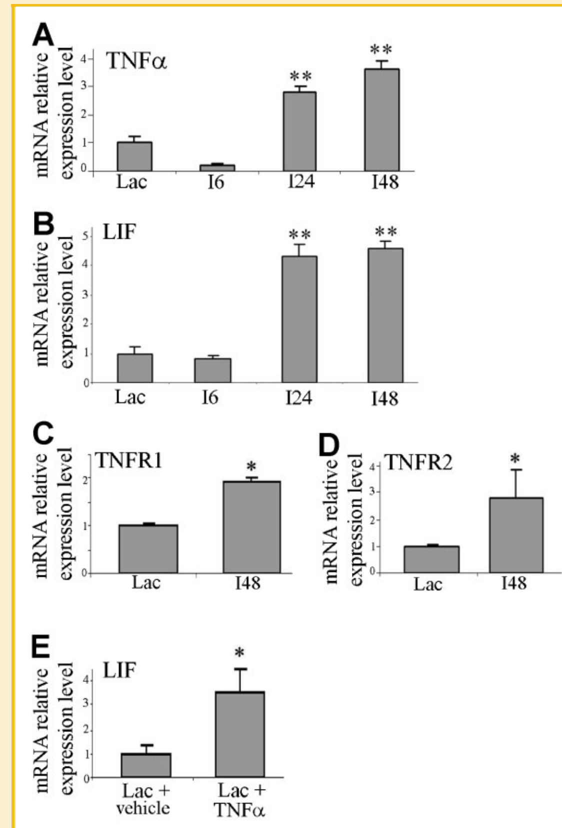

Fig. 6. LIF, TNFα and TNFR expression in the mouse mammary gland in vivo. TNFα (A), LIF (B), TNFR1 (C) and TNFR2 (D) relative to actin expression levels analyzed by qRT-PCR in involuting mammary glands at 6 h (I6), 24 h (I24), and 48 h (I48) after weaning compared to lactating (Lac) glands. Significant increase compared to lactating glands ($^*P < 0.05$; $^{**}P < 0.0005$) determined by ANOVA test. LIF relative expression levels in lactating mouse mammary glands treated with TNFα (Lac + TNFα) compared to lactating glands treated with vehicle only (Lac + vehicle). Significant increase in TNFα treated glands ($^*P < 0.05$) determined by ANOVA test (E).

level (Fig. 6E). In addition, TNFα-induced LIF protein expression in the lactating gland was confirmed by immunohistochemistry. These assays showed LIF presence in the epithelial cell cytoplasm of involuting and TNFα treated mammary glands while only weakly diffuse LIF staining was observed in sections of vehicle treated lactating glands. Figure 7A shows representative microscopic fields of both TNFα treated and control mammary glands. In addition, we investigated the ability of TNFα to induce ERK1/2 phosphorylation in vivo, by immunohistochemical analysis of pERK1/2. We observed positive staining in the nuclei and cytoplasm of epithelial cells in involuting and TNFα treated lactating glands. Figure 7B shows exemplifying microscopic fields of pERK1/2 staining in TNFα treated and control mammary glands. In addition, this Figure also shows quantification of positive nuclei in representative involuting and lactating glands treated or not with TNFα. Altogether these data suggest that in the mammary gland TNFα triggers ERK1/2 activation, which subsequently mediates LIF expression induction.

To determine whether other involution-associated phenotypic effects could be induced by TNFα treatment in vivo, we analyzed the levels of activated STAT3 by immunohistochemistry. We found positive nuclei only in involuting and TNFα-treated lactating glands (Fig. 7C), similarly to what observed when ERK1/2 activation was tested. This indicates that this treatment is able to induce multiple cellular responses associated with post-lactational involution.

DISCUSSION

LIF is a cytokine with a wide variety of cell targets. In vivo, its activity during embryo implantation, inflammation, tumor development and mammary gland involution has been extensively reported [Bhatt et al., 1991; Kellokumpu-Lehtinen et al., 1996; Kritikou et al., 2003; Schere-Levy et al., 2003; Quaglino et al., 2007;

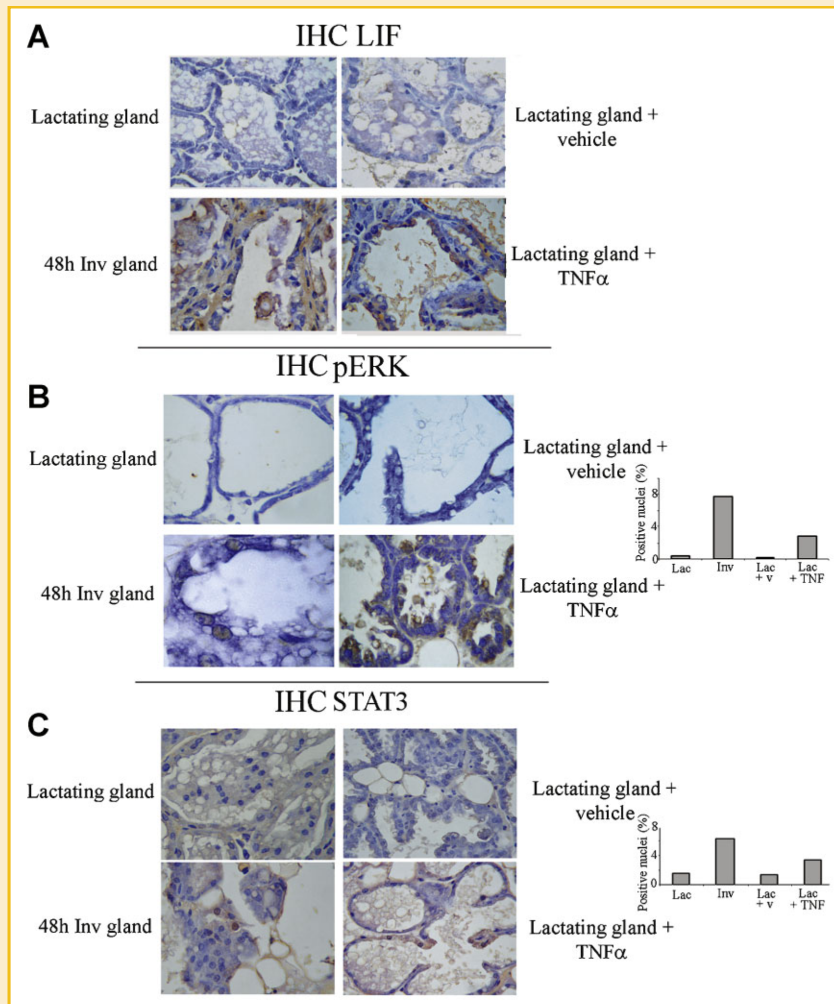

Fig. 7. Immunohistochemical analysis of LIF, phosphorylated ERK1/2 (pERK) and STAT3 in mouse mammary glands. LIF (A), pERK1/2 (B) and STAT3 (C) levels were analyzed by immunohistochemistry (IHC) in tissue sections from lactating glands, involuting glands, lactating glands treated with vehicle only and lactating glands treated with TNFα for 24 h. Cytoplasmic LIF expression was observed in epithelial cells of involuting mammary glands and TNFα-treated lactating glands (A). Nuclear and cytoplasmic pERK was observed in the involuting glands as well as in the TNFα-treated lactating glands (B). Nuclear Stat3 was observed mostly in involuting and in TNFα-treated lactating glands (C). In B and C, bar graphics show the percentage of positive nuclei in a representative sample (1,000 nuclei were counted in each case). Sections were counterstained with hematoxylin. Magnification: 100×. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

Garcia-Tunon et al., 2008]. However, the mechanisms underlying LIF expression regulation have received less attention. Here, we show that TNFα is able to induce LIF expression in mammary epithelial cells in culture and in vivo.

Our results show that ERK1/2 activation is involved in TNFα-induced LIF expression. It has been already demonstrated that TNFα activates ERK1/2 and AP-1 in tissues different from the mammary gland [Theiss et al., 2005; Srivastava et al., 2007]. However, there are no previous reports showing ERK1/2 or AP-1 involvement in induction of mammary LIF expression. Our results indicate that ERK1/2 activation is actually required for TNFα-induced LIF expression because co-treatment with a MEK inhibitor (PD98059, PD) blocked such an effect. On the other hand, inhibition of JNK activity by SP600125 (SP) during TNFα treatment enhanced LIF expression induction. Interestingly, we determined that SP treatment sustained TNFα-induced ERK1/2 activation for longer periods and that LIF over-induction elicited by this inhibitor was blocked by PD addition. Therefore, although JNK activation might be able to reduce per se LIF basal expression, over-induction generated by SP upon TNFα treatment seems to be due to the JNK inhibitory effect on ERK1/2 phosphorylation. Accordingly, previous reports support the putative opposite effect that these kinases may play on LIF expression regulation in the mouse mammary gland. It has been shown that ERK1/2 activation is present during early involution, coincidentally with LIF expression induction [Zhao et al., 2002]. Conversely, JNK activation has been observed during late pregnancy/early lactation transition [Murtagh et al., 2004], when LIF expression is very low [Schere-Levy et al., 2003].

It has been shown that TNFα triggers cell signaling through two different high affinity receptors: a 55-kDa receptor (TNF-R55 or TNFR1) and a 75-kDa receptor (TNF-R75 or TNFR2) [Dri et al., 1999]. Most reports are focused on TNFR1 mediated responses [Tartaglia

et al., 1993; Neumann et al., 1996] and only a few studies have described TNFR2-mediated activities [Vandenabeele et al., 1992; Tartaglia et al., 1993]. Specifically, in the involuting mouse mammary gland it has been indicated that TWEAK may induce apoptosis through interaction with TNFR1 [Baxter et al., 2006]. However, there are no previous reports showing a biological role for TNFR2 during this process. Our results suggest that this receptor would mediate LIF expression induction in mammary epithelial cells, although both, TNFR1 and TNFR2, might be involved in TNFα-induced ERK1/2 activation in this tissue. In addition, the data showed herein indicate that expression of both receptors is induced in the mouse mammary gland upon weaning. This observation differs from what previously reported in the rat mammary gland, in which TNFR1 mRNA is elevated during pregnancy and early lactation, declining thereafter, and TNFR2 expression increases during lactation and remains elevated through early involution [Shea-Eaton et al., 2001]. It would be interesting to find out whether the differences found in TNFR expression pattern between these species might account for alternative TNFα biological roles during mouse and rat mammary gland development.

AP-1 proteins are often the final target of signal-transducing kinase cascades triggering the expression of their corresponding regulated genes [Cavigelli et al., 1995]. Here, we show that LIF would be one of those genes. Interestingly, we found two AP-1 consensus sites in the LIF promoter region. The proximal one, at position −1866 that contains a canonic AP-1 sequence (CAAA**TGAGTCA**ACTA) coincides with the CDODN sequences used in our experiments. The other one, at −3412 bp, shows one changed base with respect to the consensus sequence (CTGC**TGAGTA**ACCAC). More experiments need to be performed to determine whether both AP-1 sites at LIF promoter regions are involved in TNFα-triggered LIF transcription induction.

Our study indicates that TNFα is able to induce LIF expression in mammary epithelial cells and that effect may be relevant in the involution process. However, we do not believe that TNFα is the only relevant factor that triggers LIF expression after lactation. As a matter of fact, our group recently demonstrated that mechanical stress—that might be exerted by milk accumulation in the alveoli at weaning—is able to induce LIF expression and secretion in mammary epithelial cells [Quaglino et al., 2009]. Therefore, early LIF expression at the end of lactation might not be due to TNFα expression. Alternatively, this factor would be relevant in maintaining high LIF levels during the first 3 days of mammary involution.

It has been demonstrated that transcriptionally, mammary postlactational regression is similar to an acute phase inflammatory response [Watson, 2009]. On the other hand, it has been shown that proinflammatory cytokines mediate the early local and systemic responses to microbial challenges. For example, when mammary gland inflammation occurs in response to bacterial infection (mastitis), small proteins, including interleukin-1 beta (IL-1β), IL-6, IL-8, and TNFα, are released by various cell types such as monocytes, neutrophils, macrophages, lymphocytes, endothelial, and epithelial cells [Sugimoto et al., 2006; Zhu et al., 2007], and play dual pathophysiological roles, as proinflammatory as well as immunoregulatory mediators [Boutet et al., 2007; Zhu et al., 2007]. The results showed herein indicate that TNFα is not only able to induce LIF expression and ERK1/2 activation in cultured mammary cells, but also in the lactating gland in vivo, together with STAT3 activation (see Fig. 7). Therefore, TNFα might be triggering involution-associated events in the healthy, as well as in the bacterially infected, mammary gland through LIF expression induction. Controlling mastitis side effects is a major concern for both, human health care and the dairy industry. Therefore, understanding the mechanisms by which inflammatory cytokines, as TNFα, triggers mammary involution may help to develop strategies to prevent the lactation interruption that often occurs upon bacterial infection of the gland.

In summary, the findings reported herein indicate that TNFα may lead to LIF expression induction during the early phase of mammary gland involution and/or upon bacterial infection through an ERK1/2-mediated pathway. In addition, TNFα ability to induce activation of AP-1 complexes and its capacity to trigger *c-fos* gene expression in SCp2 cells might imply a TNFα long lasting effect on LIF expression as well as induction of other genes involved in post-lactational epithelial cell death.

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