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TNF alpha inhibits myogenic differentiation of C2C12 cells through NF-κB activation and impairment of IGF-1 signaling pathway

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ABSTRACT

Cachexia or muscle wasting is a common condition that occurs in many chronic diseases. The wasting conditions are characterized by increased levels of TNF-α which was also known as cachectin in the past. But how TNF-α exerts its cachetic effects remains controversial. To clarify this issue, we investigated the impact of TNF-α on C2C12 cell myogenic differentiation. Our results demonstrate that myotube formation was completely inhibited by TNF-α when added to differentiating C2C12 myoblasts. The inhibitory effect of TNF-α on differentiation was accompanied by activation of NF-κB and down regulation of myogenin and Akt. Importantly, TNF-α's effect on differentiation was abolished when IGF-1 was added to the culture. IGF-1 treatment also inhibited NF-κB reporter activity and restored Akt levels. Our data suggest that TNF-α inhibits myogenic differentiation through NF-κB activation and impairment of IGF-1 signaling pathway. The reversal of TNF-α induced inhibition of myogenesis by IGF-1 may have significant therapeutic potential.

1. Introduction

Skeletal muscle atrophy is a clinical condition that occurs in various diseases such as cancer, congestive heart failure, diabetes, end stage renal failure, sepsis, and chronic obstructive pulmonary disease [1–4]. Most of these conditions are associated with elevated circulating tumor necrosis factor-α (TNF-α) [5–8]. Chronic administration of TNF-α induced weight loss and body protein redistribution in rats [9]. Treatment of cultured myotubes with TNF-α induce loss of muscle proteins [10,11]. Administration of anti-TNF IgG to tumor-bearing rats was able to reduce protein degradation rates in skeletal muscle [12].

Recent studies have identified nuclear factor-kappa B (NF-κB) as an important transcription factor involved in skeletal muscle atrophy caused by various catabolic stimuli including TNF-α [11,13–17]. In differentiating myoblasts, TNF-α induced activation of NF-κB led to reduced expression of the differentiation markers myogenin and myosin heavy chain [18]. In differentiated myotubes, transfection of a mutant I-κBα resulted in activation of NF-κB and reduction of total proteins and fast-type myosin heavy chain levels [19].

One of the important features of muscle wasting is down regulation of IGF-1/Akt signaling pathway. Overexpression of IGF-1 promotes muscle mass and strength in aging mice [20]. The purpose of the present study is to examine the role of NF-κB in the inhibition of myogenic differentiation caused by TNF-α and to determine whether IGF-1 could counteract TNF-α's negative effect on myotube formation.

2. Materials and methods

2.1. C2C12 cell culture

The mouse myoblast cell line C2C12 (Typical Culture Preservation Commission Cell Bank, Shanghai) was cultured in

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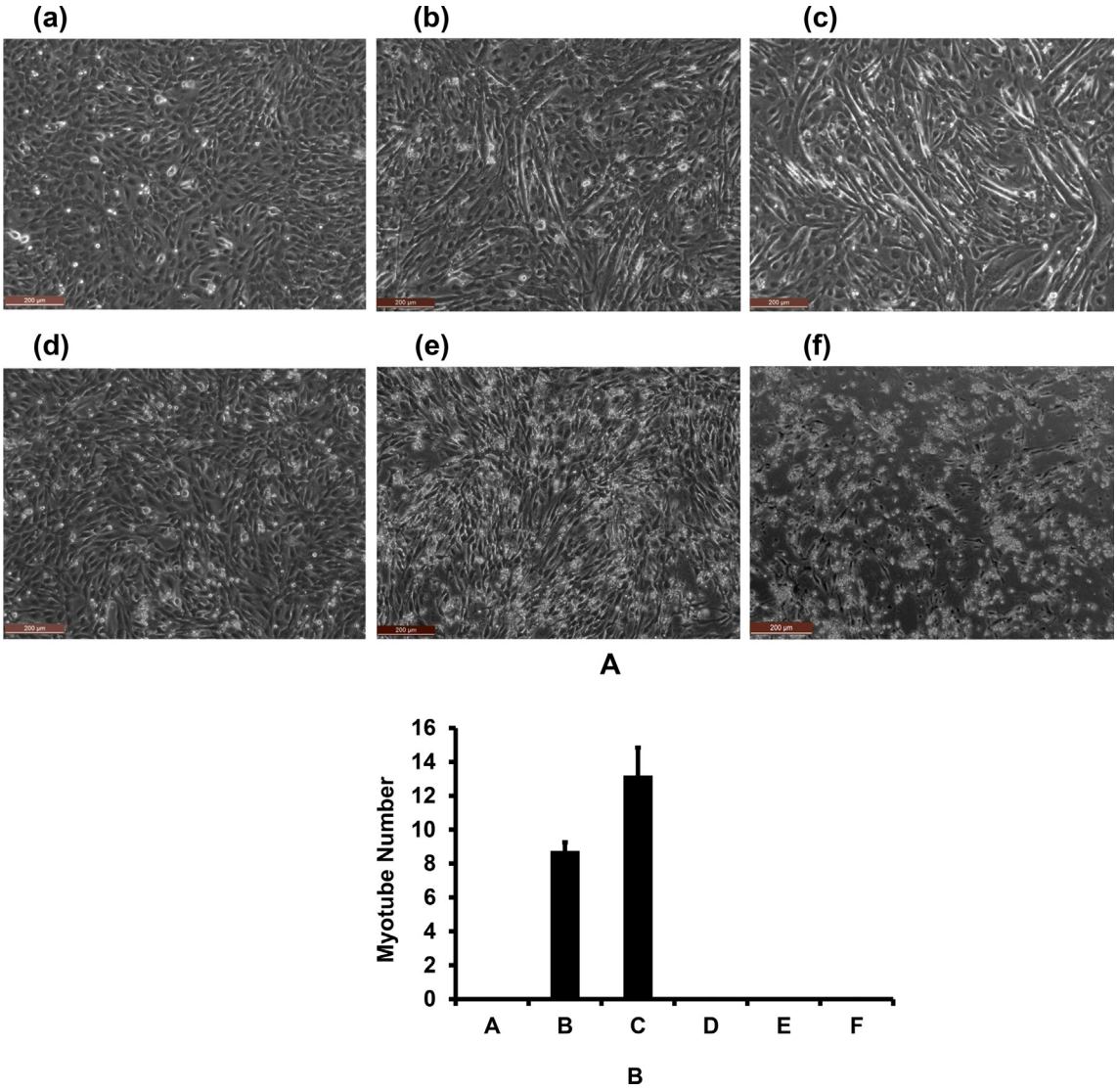

Fig. 1. The effect of TNF-α on C2C12 differentiation. A. Images of C2C12 differentiation. (a–c). C2C12 cells were cultured in differentiation medium containing 2% horse serum for 1, 4 and 6 days. (d–f). C2C12 cells were cultured in differentiation medium containing 2% horse serum in the presence of TNF-α (10 ng/ml) for 1, 4 and 6 days. B. Quantification of myotube numbers. The numbers of myotubes were counted on 4 randomly selected images for each treatment.

DMEM (Thermo Scientific) containing 10% fetal bovine serum (FBS), 100 U/ml penicillin and 100 μg/ml streptomycin in 5% CO_2 at 37 °C. To induce differentiation, DMEM containing 2% horse serum (Gibco) was added to C2C12 cells when the density of cells reached 70–80%.

2.2. Immunoblotting

Proteins extracted from C2C12 cells were separated by SDS–polyacrylamide gel electrophoresis and blotted to PVDF membranes which were subsequently incubated with primary and secondary antibodies. The following antibodies were from Cell Signaling Technology: GAPDH (1:5000), Akt (1:2000), p-Akt (1:2000) and HRP-linked anti-rabbit IgG (1:5000). Myogenin antibody was from Abcam (1:1000). The band density was quantified by ImageJ and normalized to GAPDH.

2.3. Dual-luciferase reporter assay (DLR)

C2C12 cells were seeded on 24-well plates (Corning, NY) in DMEM containing 10% FBS at a density of 30,000 cells per well and cultured overnight. Cells were switched to DMEM containing 2% horse serum, then co-transfected with 450 ng NF-κB-Luc and 50 ng RL-TK plasmid (internal control), together with Lipofectamine 2000 (Invitrogen), as previously described [21]. Three days post-transfection, cells were either treated with recombinant mouse TNF-α (10 ng/ml, R&D) for 8 h, or pretreated with recombinant mouse IGF-1 (R&D) for 4 h, followed by TNF-α and then DLR assays were performed using a luciferase assay kit (Promega, Madison, WI).

2.4. Statistical analysis

Data are presented as means ± SD. Student's *t*-test was used to determine the significance of the differences between variables. $P < 0.05$ was considered statistically significant.

3. Results

3.1. TNF-α inhibits C2C12 differentiation via impairment of IGF-1 signaling pathway

It has been shown that TNF-α induces muscle atrophy by inducing apoptosis and inhibiting myogenic differentiation

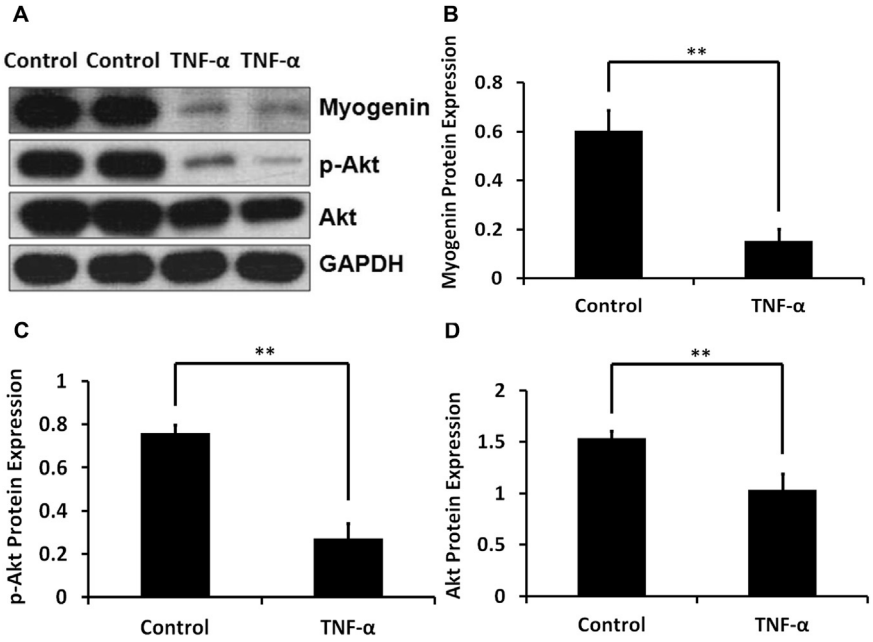

Fig. 2. The expression of Akt and myogenin in C2C12 cells. (A). The expression of myogenin, phospho- and total Akt was assessed by immunoblotting. (B–D). The bands were quantified by ImageJ and normalized to GAPDH. $^{**}P < 0.01$, n = 8.

[22–24]. In differentiating myoblasts, TNF-α inhibited myotube formation and reduced total protein content and this effect was mediated through activation of NF-κB [25]. In contrast, Chen et al. has shown that differentiating myoblasts and regenerating muscles release TNF-α which plays a pivotal role in differentiation [26]. TNF-α neutralizing antibody was able to reduce or delay the expression of differentiation markers such as p-MEF-2C, p21, myogenin and myosin heavy chain [26]. To clarify this issue, C2C12 cells were cultured in 2% horse serum with or without TNF-α for 6 days. Results showed that C2C12 cells begin to differentiate and fuse into myotubes within 4 days in the absence of TNF-α (Fig. 1). However, myotube formation was completely inhibited in the presence TNF-α (Fig. 1). Furthermore, a significant number of floating dead cells were visible after culturing the cells with TNF-α for 6 days. The inhibition of differentiation by TNF-α was accompanied by reduced protein levels of myogenin, p-Akt and total Akt, indicating that IGF-1 signaling pathway was impaired by TNF-α treatment (Fig. 2). To examine the role of IGF-1 in C2C12 differentiation, we added IGF-1 together with TNF-α to C2C12 cells cultured in differentiation medium. Results revealed that IGF-1 treatment partially restored Akt levels (Fig. 3A) and allowed C2C12 to differentiate in the presence of TNF-α (Fig. 3B,C).

3.2. The inhibition of myotube formation caused by TNF-α is mediated by NF-κB

One well-studied pathway activated by TNF-α involves signaling to NF-κB. We therefore questioned whether TNF-α induced C2C12 inhibition of differentiation is mediated by NF-κB. To address this question, we treated differentiating C2C12 cells with both TNF-α and PDTC, a specific inhibitor of NF-κB. Results showed that PDTC treatment prevented TNF-α induced inhibition of C2C12 differentiation (Fig. 3B,C), indicating that activation of NF-κB is responsible for TNF-α's effect. To confirm these findings, dual-luciferase reporter assays were performed by transfecting C2C12 cells with NF-κB-Luc, a plasmid that contain a NF-κB responsive promoter. Results showed that TNF-α induced strong activation of NF-κB reporter gene which is partially inhibited by IGF-1 (Fig. 4).

It has been shown that NF-κB overexpression caused by I-κBα silencing was able to impair insulin sensitivity by down regulation of Akt in C2C12 myotubes [27]. To determine whether NF-κB is involved in TNF-α induced inhibition of Akt expression, we treated differentiating C2C12 cells with both TNF-α and PDTC simultaneously. Our results showed that both p-Akt and total Akt levels were partially restored by PDTC (Fig. 3A), suggesting that Akt may be regulated by NF-κB through a negative feed back mechanism.

4. Discussion

Our results demonstrate that myotube formation was completely inhibited by TNF-α when added to the differentiating C2C12 cells. The inhibitory effect of TNF-α on differentiation was accompanied by activation of NF-κB and down regulation of myogenin and Akt. Importantly, TNF-α induced inhibition of myogenic differentiation was prevented by IGF-1.

IGF-1 is a unique growth factor that stimulates the proliferation of myoblasts and hypertrophy of myotubes [20,28]. Evidence suggests that circulating IGF-1 levels are reduced in wasting conditions such as sepsis and cancer [29,30]. These wasting conditions are characterized by increased levels of TNF-α which has been shown to inhibit IGF-1 transcription in both myoblasts and myotubes [31]. In rats, endotoxin injection induces a dramatic increase of TNF-α and subsequent inhibition of IGF-1 expression in tibialis anterior muscle [32]. On the other hand, recombinant IGF-1 is effective in treating muscle wasting in 129 ReJ dystrophic (dy) mice [33]. Transgenic mice overexpressing IGF-1 specifically in the skeletal muscle promote muscle regeneration by reducing the expression of inflammatory cytokines such as TNF-α and IL-1β [34]. Furthermore, muscle specific expression of IGF-1 protect muscles from angiotensin II and denervation induced muscle atrophy through activation of Akt/mTOR pathway [16,35,36]. In the present study, we provided direct evidence that IGF-1 has the ability to prevent TNF-α induced inhibition of differentiation. Taken together, the results support the rationale for using IGF-1 as a potential treatment for muscle wasting.

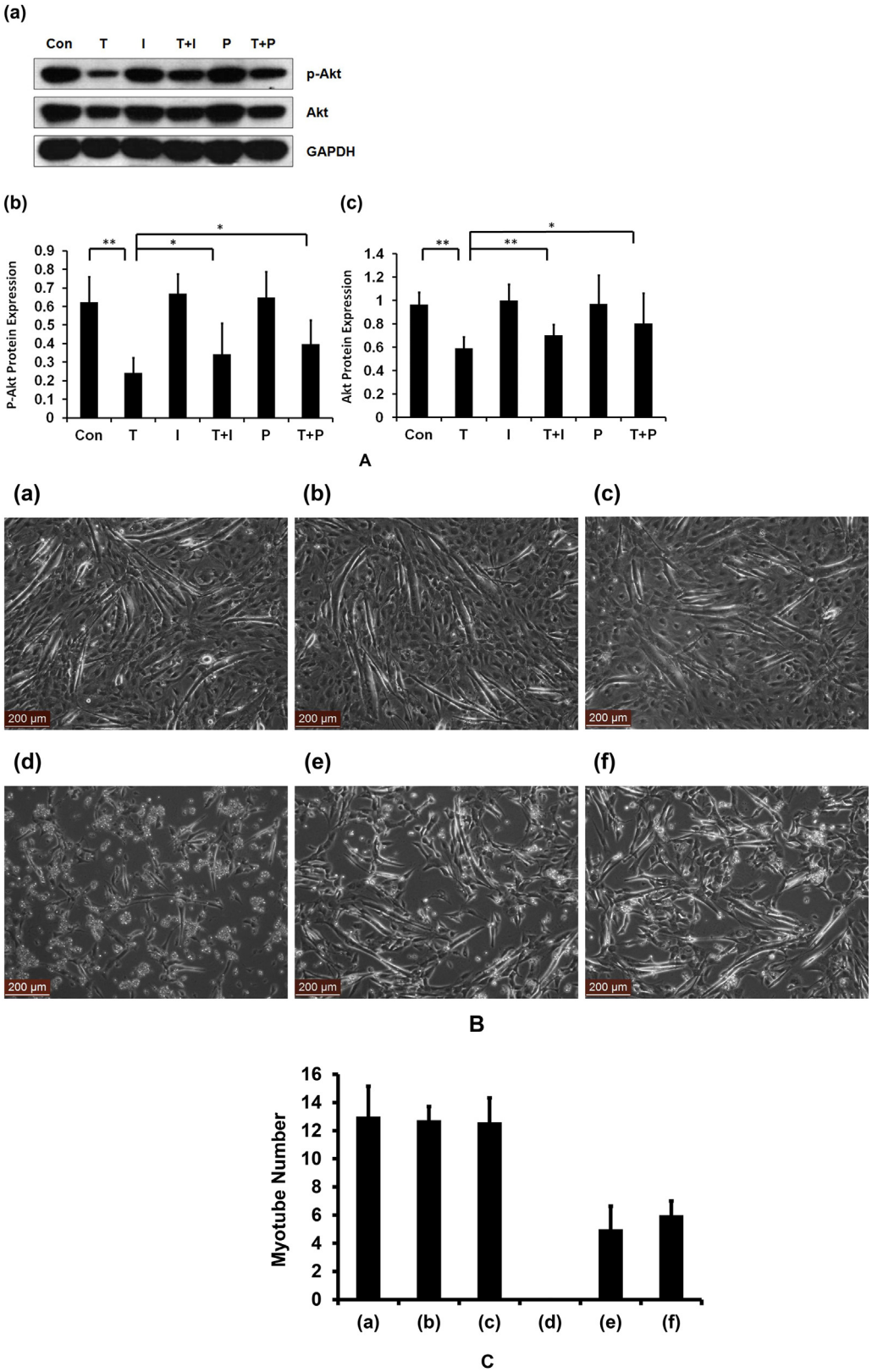

Fig. 3. The effect of IGF-1 and PDTC on Akt expression and differentiation. C2C12 cells were cultured in differentiation medium containing 2% horse serum with or without IGF-1 or PDTC treatment for 6 days. A. The expression of phospho- and total Akt was assessed by immunoblotting. (a). Immunoblot. Con: control; T: TNF-α (10 ng/ml); I: IGF-1 (30 ng/ml); T + I: TNF-α + IGF-1; P: PDTC (30 ng/ml); T + P: TNF-α + PDTC. (b–c). The bands were quantified by ImageJ and normalized to GAPDH. $^{*}P < 0.05$, $^{**}P < 0.01$, n = 5. B. C2C12 differentiation. (a). Control. (b). Cells were treated with IGF-1 (30 ng/ml). (c). Cells were treated with PDTC (30 ng/ml). (d). Cells were treated with TNF-α (10 ng/ml). (e). TNF-α (10 ng/ml) + IGF-1 (30 ng/ml). (f). TNF-α (10 ng/ml) + PDTC (30 ng/ml). C. Quantification of myotube numbers. The numbers of myotubes were counted on 4 randomly selected images for each treatment.

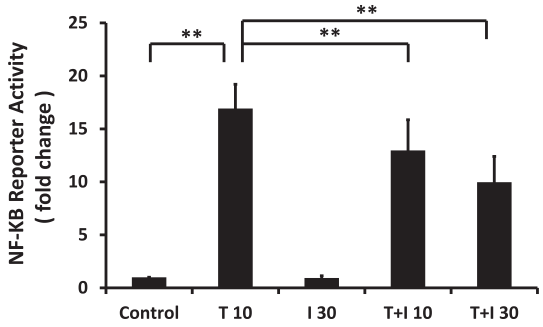

Fig. 4. The effect of TNF-α and IGF-1 on NF-κB activation in differentiating C2C12 cells. C2C12 cells cultured in differentiation medium containing 2% horse serum were co-transfected with 450 ng NF-κB-Luc and 50 ng RL-TK plasmid together with Lipofectamine 2000. Three days posttransfection, cells were treated with recombinant mouse TNF-α for 8 h, or pretreated with mouse IGF-1 for 4 h followed by TNF-α treatment. Dual-luciferase reporter assay was performed using a luciferase assay kit. T10: TNF-α, 10 ng/ml; I10: IGF-1, 10 ng/ml; I30: IGF-1, 30 ng/ml $^{**}P < 0.01$, n = 8.

It has been shown that TNF-α inhibits the expression of myogenin in myoblasts by impairing the tyrosine phosphorylation of IRS-1 and IRS-2 [37]. The phosphorylation of IRS-1 and IRS-2 is an important step toward activating the IGF-1 signaling pathway which includes down stream enzymes such as phosphatidylinositol 3-kinase (PI3K) and Akt. Akt is involved in regulation of myogenesis. Thus, the inhibition of IRS-1 and IRS-2 phosphorylation will result in down regulation of myogenin. Our results showed that TNF-α treatment led to reduced levels of Akt and myogenin, suggesting that the down regulation of myogenin caused by TNF-α may result from inhibition of Akt.

The mechanisms whereby IGF-1 inhibit NF-κB activation and prevents TNF-α's inhibitory effect on myogenesis is not clear. In astrocytes, IGF-1 prevents NF-κB nuclear translocation by activating calcineurin which is a phosphatase that dephosphorylate IκB [38]. In human colonic adenocarcinoma cells, TNF-α-induced IκB degradation was inhibited by IGF-1 through the PI3K pathway [39]. Our result showed that TNF-α induced activation of NF-κB reporter gene and down regulation of Akt can be inhibited by IGF-1 in differentiating myoblasts, suggesting that this effect may be mediated by PI3K/Akt pathway.

In conclusion, we have shown that TNF-α induced inhibition of myogenic differentiation can be prevented by IGF-1 through activation of Akt signaling pathway and inhibition of NF-κB. The reversal of TNF-α induced inhibition of myogenesis by IGF-1 may have significant therapeutic potential, because TNF-α plays a central role in various muscle wasting conditions.

Conflict of interest

None.

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Transparency document

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