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TRAF6-mediated regulation of the PI3 kinase (PI3K)–Akt–GSK3β cascade is required for TNF-induced cell survival

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ABSTRACT

We recently demonstrated that the tumor necrosis factor (TNF) receptor-associated factor 6 (TRAF6) helps maintenance of cell survival by regulating glycogen synthase kinase 3β (GSK3β) activity during TNF signaling. However, the molecular linkage between TRAF6 and GSK3β signaling is unknown. Herein, we showed that TRAF6 positively regulated cell survival by modulating PI3K–Akt–GSK3β cascades. In 3T3 cells lacking TRAF6, but not those lacking TRAF2, TNF stimulation led to prolonged hyperphosphorylation of Akt, which coincided with the activation of upstream PI3K. Pharmacologically blocking PI3K significantly inhibited Akt and GSK3β phosphorylation. Importantly, PI3K inhibition rescued cell death in TRAF6-null 3T3 cells. These data suggested TRAF6 regulates TNF-mediated cell survival through PI3K–Akt–GSK3β cascades.

The discovery of the tumor necrosis factor (TNF) receptor-associated factors (TRAFs) has provided a long sought-after link between pro-inflammatory cell surface receptors, including members of the TNF receptor (TNFR) superfamily, and the distal signaling machinery in the cell's interior. TRAFs operate by activating transcription factors, such as NF-κB, AP-1, and NFAT [1–3]. The six known mammalian TRAFs (TRAF1–TRAF6) were classified as a gene family based on a conserved TRAF domain at the C-terminus, which mediates self-association and interactions with receptors and other signaling proteins [4]. TRAF family proteins also feature an N-terminal zinc-binding domain that is believed to activate several mitogen-activated protein kinase (MAPK) kinase kinases (MAPKKKs), including TAK1, ASK1, and NIK [5,6]. Recently, a novel member of the TRAF family, TRAF7, was designated as a TRAF based on homology in the RING and zinc finger domains to the other TRAF proteins [7].

TRAF6 is unique among TRAF family members in that it also participates in interleukin-1 receptor (IL-1R)/Toll-like receptor (TLR) superfamily signaling [8,9] through the MyD88-IRAK cascade. Through this pathway, TRAF6 activates the NF-κB and MAPK cascades [10], thereby playing an essential role in innate immune responses. Furthermore, accumulating evidence indicates that TRAF6 regulates various biological processes related to bone metabolism, skin appendices, neuron development, inflammation, and adaptive immunity [3,8,10].

Recently, we demonstrated a novel role for TRAF6 in TNF-induced NF-κB activation and protection from cell death [11]. TRAF6 suppressed cell death by inhibiting ROS accumulation, which in turn prevented sustained activation of the JNK pathway. Interestingly, we found that TRAF6 deficiency led to constitutive phosphorylation and inactivation of glycogen synthase kinase 3β (GSK3β), which subsequently downregulated NF-κB transcriptional activity.

Herein, we sought to unravel the unknown molecular mechanism by which TRAF6 regulated the GSK3β cascades in TNF signaling. We characterized the response of TRAF6-deficient 3T3 ($T6^{-/-}$ 3T3) cells to TNF and we found that TRAF6 regulated TNF-induced cell survival by modulating the PI3K–Akt–GSK3β cascade.

Materials and methods

Cell culture and reagents. 3T3 cells from wild-type (WT), $TRAF2^{-/-}$, and $TRAF6^{-/-}$ mice were designated WT, $T2^{-/-}$, and $T6^{-/-}$ 3T3, respectively. Theses cells were established in culture from E14.5 embryos using a standard 3T3 protocol [11,12] and were maintained under standard conditions [Dulbecco's modified Eagle's medium (DMEM; GIBCO, Grand Island, NY), 10% fetal bovine serum (Hyclone, Logan, UT), 100 U/ml streptomycin, and 100 U/ml penicillin (GIBCO) at 37 °C, 5% CO_2]. Recombinant murine TNF was purchased from R&D Systems (Minneapolis, MN). Antibodies to phosphorylated GSK3β (Ser9), GSK3β, phosphorylated Akt (ser473), and Akt were purchased from Cell Signaling Technology (Beverly, MA). Antibodies to p85 and β-actin were purchased from Upstate Biotechnology (Lake Placid, NY) and Sigma (St. Louis, MO), respectively.

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Western blot analysis. After stimulation with recombinant murine TNF, cells were lysed in 1% Nonidet P-40 lysis buffer [50 mM Hepes (pH 7.4), 150 mM NaCl, 2 mM EDTA, 1% Nonidet P-40, and protease inhibitors]. The cell lysates were resolved by SDS–PAGE and analyzed by Western blotting with the specific antibodies listed above. For some experiments, cells were pre-incubated with the PI3K inhibitor LY294002 (Sigma) prior to TNF stimulation.

Immunoprecipitation and PI3K assay. 3T3 cells from WT, TRAF2$^{-/-}$, and TRAF6$^{-/-}$ mice were treated with TNF for the indicated time periods. The cells were lysed and incubated with anti-p85 antibody and protein A–agarose beads for 1 h. Immunoprecipitates were washed and evaluated for PI3 K activity by competitive ELISA (Echelon Biosciences, Salt Lake City, UT) as described [13]. Briefly, the bead-bound immunoprecipitated enzyme was incubated with phosphatidylinositol (4,5)-bisphosphate [PI(4,5)P_2] substrate (100 pmol) in kinase reaction buffer [4 mM $MgCl_2$, 20 mM Tris (pH 7.4), 10 mM NaCl, and 25 μM ATP] for 2 h at room temperature with shaking. The supernatant was then incubated with a PI(3,4,5)P_3 detector protein for 1 h at room temperature, and the reaction mixtures were transferred to PI(3,4,5)P_3-coated detection plates and incubated for 1 h at room temperature. After washing with TBST [150 mM NaCl, 10 mM Tris (pH 7.5), and 0.05% (vol/vol) Tween 20], secondary detection reagent (supplied with the kit) was added, plates were washed again, developing solution (supplied with the kit) was added, and the extent to which the PI(3,4,5)P_3 detector protein bound to the plate was determined by measuring the absorbance at 450 nm.

Cell death assay. After treatment with TNF alone or TNF in combination with cycloheximide (Sigma) and/or LY294002 for the indicated time periods, cells were harvested, stained with propidium iodide (Sigma), and analyzed with a flow cytometer (FACS Calibur; BD Biosciences, Rockville, MD).

Data analysis. The levels of phosphorylated Akt or GSK3β were determined using the software, Science Lab Image Gauge V4.0 software (Fujifilm; Mississauga, Ont., Canada). With regard to PI3K assay results, the activity of PI3K was expressed as a percentage of the activity measured in control-treated cells, as suggested by the manufacturer (Echelon Biosciences).

Results

TNF-induced hyperphosphorylation of Akt in T6$^{-/-}$ 3T3 cells

TRAF6 plays a role in cell survival maintenance by regulating GSK3β activity during TNF signaling [11], but the mechanism underlying how TRAF6 controls GSK3β is unknown. Since GSK3β is a downstream target of Akt [14], we examined whether loss of TRAF6 resulted in augmented Akt phosphorylation. As shown in Fig. 1A, transient phosphorylation of Akt increased slightly in WT and T2$^{-/-}$ cells, but T6$^{-/-}$ cells exhibited hyperphosphorylation of Akt. Moreover, after cells were exposed to TNF for 2–8 h, Akt phosphorylation was sustained in T6$^{-/-}$, but not in WT and T2$^{-/-}$ cells (Fig. 1B). These results suggested that TRAF6 deficiency led to hyperphosphorylation of Akt, which might cause phosphorylation (i.e., inactivation) of GSK3β.

PI3K activity is enhanced in T6$^{-/-}$ 3T3 cells

PI3K is upstream of Akt, because its product, PI(3,4,5)P_3, stimulates Akt activation [15]. To determine whether hyperphosphorylated levels of Akt in T6$^{-/-}$ cells were influenced by PI3K, we assessed PI3K activity by competitive ELISA. Transient and longer-lasting TNF stimulation significantly enhanced PI3K activity in T6$^{-/-}$ cells compared to WT or T2$^{-/-}$ cells (Fig. 2A and B). However, PI3K expression in T6$^{-/-}$ cells was not significantly different than in WT or T2$^{-/-}$ cells (data not shown). This observation indicated that TRAF6 deficiency increased the activity of PI3K.

PI3K inhibition decreased phosphorylation of Akt and GSK3β

To determine whether the enhanced PI3K activity in T6$^{-/-}$ cells caused Akt phosphorylation and led to GSK3β hyperphosphorylation, we treated T6$^{-/-}$ cells with a pharmacological inhibitor of PI3K, LY294002. Treatment with the inhibitor markedly deactivated PI3K activity (Fig. 3A). Consistent with the decreased PI3K activity, LY294002 significantly inhibited basal levels of Akt and GSK3β phosphorylation, respectively (Fig. 4B) without altering total Akt or GSK3β levels. These results suggested that hyperactivation of the PI3K–Akt pathway led to GSK3β inactivation in T6$^{-/-}$ cells.

PI3K inhibition rescued TNF-induced cell death in T6$^{-/-}$ cells

If hyperactivation of the PI3K-Akt pathway in T6$^{-/-}$ cells inactivated the GSK3β that was leading to TNF-induced cell death, then PI3K inhibition should attenuate cell death. To test this hypothesis, we compared TNF-induced cell death in T6$^{-/-}$, T2$^{-/-}$, and WT cells. Consistent with a previous report [11], TNF induced rapid cell death of T6$^{-/-}$ and T2$^{-/-}$ cells and this susceptibility significantly increased in the presence of cycloheximide. Interestingly, pretreatment of T6$^{-/-}$ cells with LY294002 significantly reduced cell death in T6$^{-/-}$ cells, but not in T2$^{-/-}$ or WT cells, indicating that TRAF6

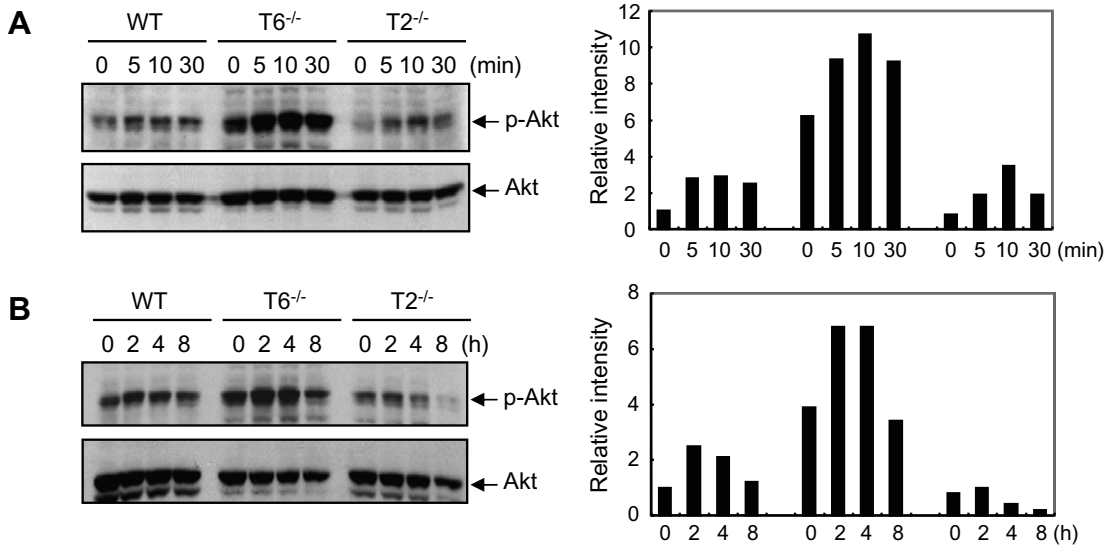

Fig. 1. TRAF6 deficiency led to hyperphosphorylation of Akt. (A) WT, T6$^{-/-}$, and T2$^{-/-}$ 3T3 cells were stimulated with TNF (30 ng/ml) for the indicated times. Levels of phosphorylated Akt (p-Akt) were determined by immunoblotting. The membranes were reblotted with antibody to Akt as a control. The relative intensity in the stimulated cells compared to WT untreated cells is shown in the right panel. (B) Cells were treated as in (A), except for the indicated times. The relative intensity in the stimulated cells compared to WT untreated cells is shown in the right panel.

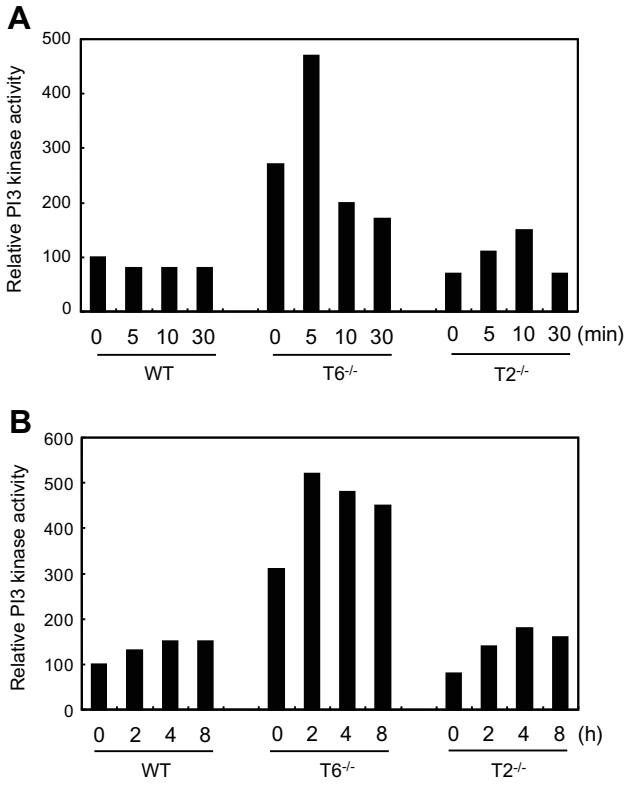

Fig. 2. TNF stimulation resulted in hyperactivation of PI3K in $T6^{-/-}$ cells. (A) WT, $T6^{-/-}$, and $T2^{-/-}$ 3T3 cells were stimulated with TNF (30 ng/ml) for the indicated times. PI3K was immunoprecipitated and its activity was analyzed as described in Materials and methods. Data were quantified and expressed as a percentage of the activity measured in control ells. (B) Cells were treated as in (A), except for the indicated times.

acted as a positive regulator of cell survival by modulating the PI3K–Akt–GSK3β cascade.

Discussion

In the present study, we characterized the mechanism underlying how TRAF6 controls GSK3β during TNF signaling. Our study

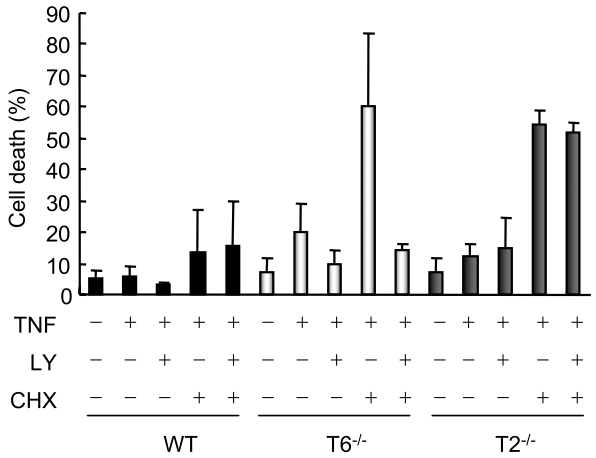

Fig. 4. Rescue of cell death in $T6^{-/-}$ 3T3 cells by PI3K inhibition. (a) WT, $T6^{-/-}$, and $T2^{-/-}$ 3T3 cells were exposed to TNF (30 ng/ml) in combination with cycloheximide (5 μg/ml) and/or LY294002 (10 μM) for 12 h, harvested, stained with propidium iodide, and analyzed by flow cytometry to determine the extent of cell death. Data indicate means ± SD of three independent experiments.

demonstrated that increased GSK3β phosphorylation was mediated by enhanced PI3K–Akt signaling activation in $T6^{-/-}$ cells.

The central role of PI3K–Akt pathway in receptor-mediated regulation of cell survival has well been well documented in a variety of cell types [16–18]. Moreover, many reports have elucidated important roles for the Akt signaling pathway in cell survival associated with several types of cancer [19–21]. However, consistent with our data, recent evidence suggested that PI3K does not always support cell survival: it instead promotes cell death under specific experimental conditions. For example, PI3K inhibition using specific inhibitors or dominant negative mutants suppressed cell death mediated by arsenite [22], hypoxia [23], glucose deprivation [24], or serum withdrawal [25]. Therefore, it is likely that the PI3K–Akt pathway differentially promotes cell survival or cell death, depending on the cellular contexts or stimuli.

What is the underlying purpose behind TRAF6-mediated control of the PI3K–Akt–GSK3β pathway? It is feasible that, in normal or cancer cells, the active form of GSK3β is essential for cell survival through NF-κB activation [26,27]. In such circumstances, the PI3K–Atk signals upstream of GSK3β needs to be kept under a strict control because, upon TNF stimulation, improperly activated PI3K–Akt

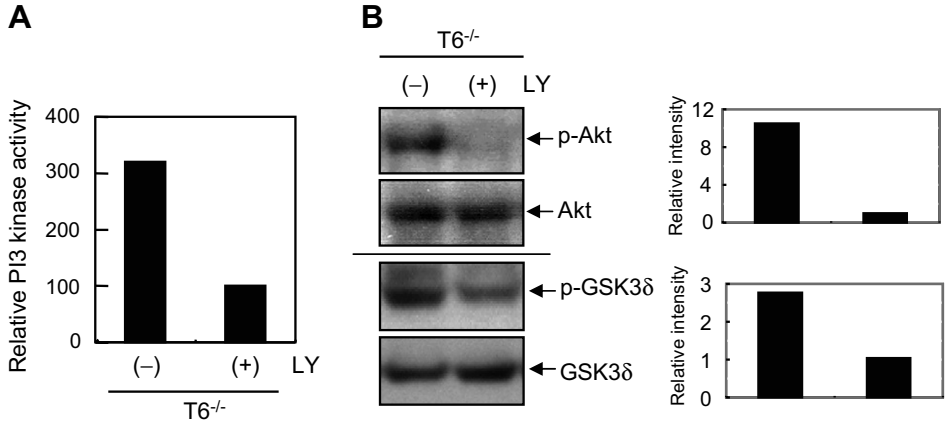

Fig. 3. PI3K-dependent hyperphosphorylation of Akt and GSK3β in $T6^{-/-}$ cells. (A) $T6^{-/-}$ 3T3 cells were treated with PI3K inhibitor (LY294002), and PI3K activity was measured as in Fig. 2A. (B) Cells were treated as in (A), except that levels of phosphorylated Akt (p-Akt; upper panel) and GSK3β (p-GSK3β; lower panel) were determined by immunoblotting. The membranes were reblotted with antibodies against Akt or GSK3β as controls. The relative intensity in the stimulated cells compared to LY294002-treated cells is shown in the right panel.

signaling might switch from promoting cell survival to promoting cell death as a result of GSK3β inactivation. Recently, consistent with our observations, specific deletion of TRAF6 in T cells increased their basal levels of phosphorylated Akt, a response that was apparently mediated by increased PI3K activation [28]. Further studies exploring how TRAF6 regulates PI3K activity during TNF signaling remain to be elucidated.

Acknowledgments

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