Dual regulation of soluble tumor necrosis factor-α induced activation of human monocytic cells via modulating transmembrane TNF-α-mediated 'reverse signaling'

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Abstract. Transmembrane tumor necrosis factor-α (mTNF-α) is known to be the precursor of soluble TNF-α (sTNF-α). mTNF-α can act as a ligand on the TNF receptor- (TNFR)-bearing cell through 'forward signaling' or as a receptor on the TNF producing cell through 'reverse signaling'. In the current study, we investigated the role of mTNF-α-mediated reverse signaling in regulating sTNF-α-induced activation of human monocytic U937 cells. We demonstrated that pretreatment with sTNFRI, for inducing reverse signaling through mTNF-α, sensitized U937 cells to sTNF-α stimulation, as evidenced by an increase in reactive oxygen production and mRNA levels of proinflammatory cytokines (TNF-α, IL-1ß, and IL-8) in these cells. Further experiments revealed that IκB-α degradation was increased in the monocytic cells primed with sTNFRI, implying that reverse signaling of mTNF-α sensitizes U937 cells via an NF-κB-dependent mechanism. On the other hand, binding of sTNFRI to mTNF-α after sTNF-α-induced activation of U937 cells reduced mRNA stability (half-life) of IL-1ß and IL-8. The involvement of reverse signaling in the process was verified by using a mutated form of mTNF-α lacking the majority of the cytoplasmic domain. Our results clearly showed that enhanced mRNA degradation of the cytokines occurred only in U937 cells transfected with a wild-type mTNF-α, but not in those cells transfected with the mutant mTNF-α. Taken together, these data suggest that reverse signaling through mTNF-α may exert a double role in modulating sTNF-α bioactivity. It is positive when reverse signaling occurs prior to sTNF-α stimulation, while it is negative when reverse signaling occurs after the sTNF-α signal. Thus, our findings strengthen a role of mTNF-α-mediated reverse signaling in the regulation of immune-inflammatory response and control of inflammatory reaction.

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Introduction

Tumor necrosis factor-α (TNF-α) is a pleiotropic proinflammatory cytokine with a diverse range of biological activities, and is produced by activated monocytes and macrophages as well as other cell types (1). The precursor of mature TNF-α is called transmembrane TNF-α (mTNF-α), which is a 26-kDa cell surface transmembrane type II polypeptide. TNF-α converting enzyme (TACE) proteolytically cleaves the C-terminal portion of mTNF-α to release its extracellular segment as a 17-kDa soluble TNF-α (sTNF-α) (2,3). In contrast to sTNF-α, mTNF-α exerts its biological functions in a cell-to-cell contact fashion. Besides cytotoxic activity, several immunological functions of mTNF-α, which is distinct from sTNF-α, have been reported (4). mTNF-α expressed on activated $CD4^+$ T cells provides a costimulatory signal for B cell activation and IL-4-dependent Ig synthesis (5). Furthermore, mTNF-α is also involved in other biological processes, such as the activation of polyclonal B cells induced by human immune deficiency virus-1 (HIV-1) or human T cell leukemia virus (HTLV) I-infected $CD4^+$ T cells (6,7), production of IL-10 from monocytes (8), and expression of ICAM-1 by endothelial cells (9). All of these biological effects of mTNF-α are likely mediated through both TNF receptor types, in which mTNF-α is the primary activating ligand of TNF receptor II (TNFRII) (10).

Recent studies revealed that mTNF-α acts not only as an activating ligand, but also as a receptor that transmits reverse signals into the effector cells. Activation of mTNF-α with

Table I. RT-PCR primers and amplified products of *TNF-α, IL-1ß, IL-8,* and *ß-actin* genes.

Gene	Forward primer	Reverse primer	Fragment (bp)
TNF-α	5'-gcggatccatgagcactgaaagcatgatcc-3'	5'-gctctagatcacagggcaatgatcccaaag-3'	700
IL-1ß	5'-ggcatccagctacaaatctc-3'	5'-cttgagaggtgctgatatac-3'	600
IL-8	5'-atttctgcagctctgtgtgaa-3'	5'-tgaattctcagccctcttcaa-3'	250
ß-actin	5'-atctggcaccacacctccta-3'	5'-ctccttaatgtcacgcacga-3'	400

antibody (Ab) against TNF-α or with soluble TNF receptor (sTNFR) was found to lead to the mobilization of intracellular calcium (7,11) and induction of IL-2 and IFN-γ (7). Reverse signaling of mTNF-α was also shown to modulate anti-CD3-triggered T cell cytokine transcription of IFN-γ and IL-4 in mice (12). It was also demonstrated that stimulation of mTNF-α with anti-TNF-α Ab reversely induced E-selectin expression on different cell types, including HTLV-I-infected T cell lines, activated human $CD4^+$ T cells, and transfected HeLa cells (13).

TNF-α is a multifunctional cytokine which plays a central role in inflammation. Owing to the rapid production by macrophages in response to infectious challenges, sTNF-α augments the phagocytic and cytotoxic actions of macrophages and triggers the synthesis and secretion of proinflammatory mediators, including IL-1, GM-CSF, TNF itself, and other cytokines, to amplify its range of actions (14,15). However, the role and importance of the reverse signaling via mTNF-α in inflammation remain largely unknown at the present time. The reverse signaling of mTNF-α was reported to serve as a silencing signal rather than a positive regulator of inflammation in the literature (16,17). Reverse signaling through mTNF-α rendered monocytes and macrophages resistant to lipopolysaccharide (LPS), as indicated by down-regulating LPS-induced sTNF-α, IL-6, IL-1, and IL-10 (16). When prestimulated with TNF monoclonal antibodies or sTNFR, monocytic cells were resistant not only to LPS, but also to other inflammatory agents like zymosan (17). We observed in this study that mTNF-α both positively and negatively regulated sTNF-α-induced activation of the human monocytic cell line U937. Pre-activation of mTNF-α with sTNFRI increased the sensitivity of U937 cells to sTNF-α stimulation, whereas stimulation of mTNF-α with sTNFRI 4 h after sTNF-α-induced U937 cell activation remarkably enhanced mRNA degradation of inflammatory cytokines, suggesting a dual role of the mTNF-α reverse signaling in the regulation of sTNF-α bioactivity in U937 cells.

Materials and methods

Cell culture and stimulus. The human monocytic leukemia cell line U937 was cultured in Dulbecco's modified Eagle's medium (DMEM) (Invitrogen, Gaithersburg, MD, USA), supplemented with 10% heat-inactivated fetal calf serum (FCS, Invitrogen), 100 U/ml of penicillin, and 100 μg/ml of streptomycin at 37˚C in 5% CO_2. sTNF-α was purchased from Peprotech (London, UK).

Construction, expression, and purification of sTNFRI. Human cDNA coding for the extracellular region of sTNFRI (275-772) was amplified by RT-PCR in 30 cycles (94˚C for 30 sec, 60˚C for 60 sec, and 72˚C for 60 sec). A pair of primers used was: P1, 5'-CAGGATCCGATAGTGTGTGTCCCCAAG-3' and P2, 5'-CCCAAGCTTCTCAGTGCCCTTAACATTC-3'. The cDNA for sTNFRI was then cloned into the BamHI and *Hin*dIII cloning site of the expression vector pET-28a (+) (18). sTNFRI was expressed and purified by Ni2+-NTA resin up to 95% purity. Endotoxin was removed using a Detoxi-Gel endotoxin-removing gel column (Pierce, Rockford, USA) according to the manufacturer's instructions. Residual endotoxin concentration was <0.2 U/mg.

Measurement of reactive oxygen production. U937 cells ($1x10^6$/ml) were pretreated with varying concentrations of sTNFRI (0.1, 1, or 10 μg/ml) for 30 min for inducing the mTNF-α reverse signaling, then unbound sTNFRI was washed away, and sTNF-α (10 U/ml) were added to stimulate U937 cells for an additional 2 h. The production of reactive oxygen in supernatants was then determined with the reactive oxygen kit (Jianchen Company, Nanjing, P.R. China) according to the manufacturer's instructions. Data are presented as the mean ± SD.

Analysis of cytokine mRNA expression by RT-PCR. Cellular RNA was isolated using the Tripure isolation reagent (Roche, Indianapolis, IN, USA) according to the manufacturer's instructions. Total RNA (1 μg) was reversely transcribed to cDNA by using the Perkin Elmer GeneAmp RNA PCR kit (Foster City, CA, USA). The primers used for *TNF-α, IL-1ß, IL-8* and *ß-actin* gene amplification are shown in Table I. Each PCR mixture (in a total of 50 μl) contained 2 mM $MgCl_2$, 800 μM each dNTP, 1 μM each primer, 50 ng of plasmid DNA, and 2 units of Taq polymerase (Takara, Japan). The reaction was performed in 30 cycles (94˚C, 30 sec; 58˚C, 30 sec; and 72˚C, 1 min). Amplification products (TNF-α, IL-1ß, IL-8 and ß-actin) were separated by electrophoresis on 1% agarose gels. The primers (Table I) were chemically synthesized with a DNA synthesizer (Bioasia, P.R. China).

Plasmid construction and transient transfection. The wild-type TNF-α cDNA was cloned into the BamHI and XbaI cloning sites of the eukaryotic expression vector pCDNA 3.0 (Invitrogen). The sequence encoding for the majority of the cytoplasmic segment from the position -73 to -47 of TNF-α was deleted by PCR, for which the wild-type TNF-α cDNA as the template and a pair of primers 5'-GCGGATCCATGA GCACTTTGTTCCTCAGCCTCTTCTCC-3' and 5'-GCTCT

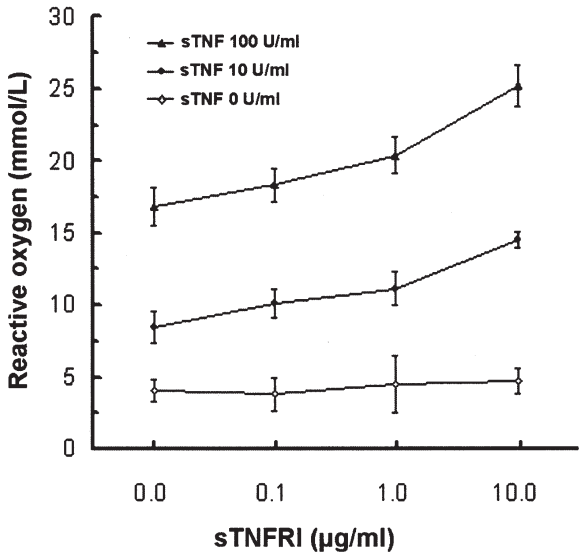

Figure 1. Increment of sTNF-α induced reactive oxygen production in U937 monocytic cells via pre-activating the reverse signaling of mTNF-α. U937 cells (1x10^6/ml) were pretreated with varying concentrations of sTNFRI, as indicated, for activating the mTNF-α reverse signaling for 30 min. The unbound sTNFRI was washed away, and sTNF-α at 10 U/ml or 100 U/ml was then added to the culture to stimulate U937 cells for an additional 2 h. At the end of incubation, cell culture supernatants were collected for measuring reactive oxygen production. Results shown are the mean ± SD of 3 independent experiments performed with duplicate samples.

AGATCACAGGGCAATGATCCCAAAG-3' were used. The mutant TNF-α cDNA was amplified in 30 cycles (94˚C, 30 sec; 58˚C, 60 sec; and 72˚C, 1 min), and accuracy of the sequence was confirmed by sequencing, and then the mutant TNF-α cDNA was cloned into BamHI and XbaI cloning sites of pCDNA3.0.

U937 cells (4x10^6/ml) were transiently transfected with 10 μg of plasmid at 100 μs/300 V using an electroporator (Eppendorf AG, Hamburg, Germany). After 48 h of transfection, the transfected cells were stimulated with 100 U/ml sTNF-α for 4 h, washed 3 times with PBS, and then treated with 10 μg/ml sTNFRI. The cells were harvested at different time points for RNA isolation and RT-PCR analysis. The transfection efficiency was controlled by co-transfection with 1 μg of pEGFP-N1.

Western blot analysis. Total protein (50 μg) in cell lysates were separated on a 10% SDS-polyacrylamide gel and electroblotted to polyvinylid-denedifluoride (PVDF) membrane (Boehringer Mannheim GmbH, Germany) for 1 h at 20 V using a semidry blotting apparatus (Trans-blot SD; Bio-Rad, Richmond, CA, USA). The membranes were blocked with 5% nonfat dry milk in PBS-Tween-20 (0.05%) overnight at 4˚C and probed with a rabbit polyclonal antibody, such as anti-IκBα at 1:1000 (Santa Cruz Biotechnology, Santa Cruz, CA, USA) or anti-ß-actin at 1:1000, or with a mouse mAb anti-human TNF (Biosource) at 1:1000 for 2 h at room temperature, followed by incubation with a goat anti-rabbit polyclonal antibody conjugated with horseradish peroxidase (HRP) at 1:10,000 (Zhong Shan, P.R. China) or with a goat anti-mouse IgG coupled to HRP at 1:10,000 for 1 h at room temperature. The membrane blotted with proteins synthesized *in vitro* was incubated with streptavidin-POD at a final concentration of 0.1 U/ml for 30 min. The immunoreactivities of blots were detected by using a BM chemiluminescence blotting kit (Roche). Namely, the membrane was incubated in 1 ml of the chemiluminescence substrate mixture for 1-10 min and then exposed to X-ray film (Kodak, Japan).

Results

Increment of sTNF-α induced reactive oxygen production in U937 cells by pre-activating reverse signaling through mTNF-α. We set out to determine the endogenous mTNF-α expression on U937 cells by flow cytometry (FACS), and we found that mTNF-α positive cells were ~45% of the total U937 cells (data not shown). Since sTNF-α is an activator for monocyte and macrophage activation, including enhanced phagocytosis, killing of microbes, and respiratory burst (19), we assessed whether the pre-activation of mTNF-α reverse signaling with sTNFRI affected sTNF-α-induced reactive oxygen production. As shown in Fig. 1, the unstimulated U937 cells generated a basal level of reactive oxygen (≤5 mmol/l), and sTNF-α-induced U937 cells released large amounts of reactive oxygen ($p<0.01$) in a concentration-dependent fashion, which peaked at 100 U/ml of sTNF-α. The treatment of sTNFRI alone did not increase reactive oxygen production; however, the pre-activation of mTNF-α reverse signaling with sTNFRI dose-dependently enhanced sTNF-α-induced reactive oxygen production in U937 cells compared to the cells treated with sTNF-α alone ($p<0.01$). These results indicated that mTNF-α reverse signaling pre-triggered by sTNFRI rendered U937 cells more susceptible to sTNF-α stimulation. As 10 μg/ml of sTNFRI showed the maximal effect on reactive oxygen production in U937 cells, we used this concentration for inducing mTNF-α reverse signaling in the following experiments.

Enhancement of sTNF-α induced mRNA expression of TNF-α, IL-1ß, and IL-8 in U937 cells by pre-activation of reverse signaling through mTNF-α. Another important action of sTNF-α in monocytes and macrophages is to upregulate proinflammatory cytokine expression, such as IL-1, IL-8, and TNF-α (20,21). Therefore, we investigated whether mTNF-α-mediated reverse signaling also increased sTNF-α-induced mRNA expression of proinflammatory cytokines by RT-PCR analysis. As illustrated in Fig. 2, sTNFRI alone did not change the basal mRNA levels of TNF-α, IL-1ß and IL-8 (lane 2 vs. lane 1). In contrast, sTNF-α alone increased gene expression of the proinflammatory cytokines, and the effect of sTNF-α in this regard was maximal at 100 U of sTNF-α in U937 cells (lane 5 vs. lane 3). Not surprisingly, the pre-activation of mTNF-α reverse signaling with sTNFRI significantly enhanced sTNF-α-induced transcription of the proinflammatory cytokines, as compared to that induced by sTNF-α alone (lane 6 vs. lane 5 and lane 4 vs. lane 3), indicating that mTNF-α reverse signaling primed U937 cells to sTNF-α-induced activation.

Augmentation of sTNF-α induced IκB-α degradation in U937 cells by pre-activation of reverse signaling through mTNF-α.

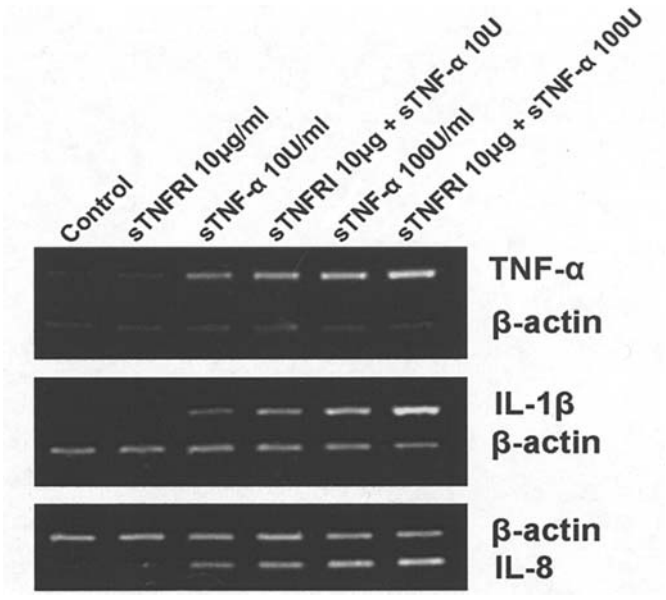

Figure 2. Augmentation of sTNF-α induced mRNA expression of proinflammatory cytokines in monocytic U937 cells via pre-activating the reverse signaling of mTNF-α. U937 cells ($1x10^6$/ml) were pretreated with 10 μg/ml sTNFRI for 30 min, and the cells were then stimulated with 10 U/ml or 100 U/ml of sTNF-α for an additional 3 h. Cells treated with sTNFRI or sTNF-α alone were used as controls. The mRNA levels of proinflammatory cytokine expression (top: TNF-α; middle: IL-1ß; bottom: IL-8) were analyzed by RT-PCR. The data presented are representative of 3 separate experiments.

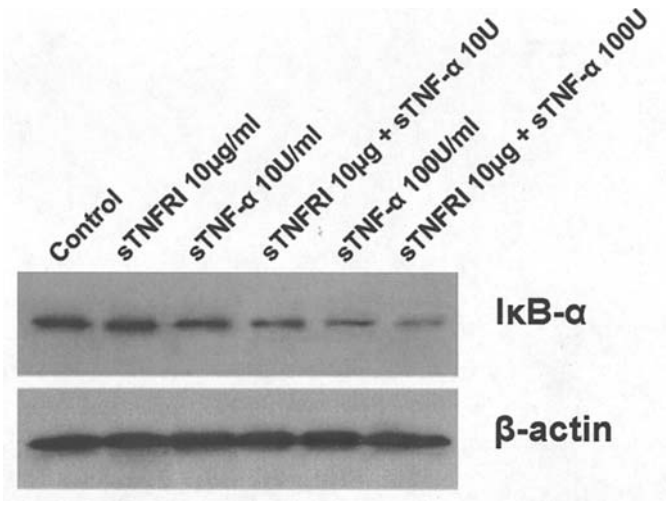

Figure 3. Promotion of sTNF-α induced IκB-α degradation in U937 monocytic cells via pre-activating the reverse signaling of mTNF-α. U937 cells ($1x10^6$/ml) were pretreated with 10 μg/ml sTNFRI for 30 min, and the cells were then stimulated with the indicated concentrations of sTNF-α for an additional 30 min. Cells treated with sTNFRI or sTNF-α alone were used as controls. Cellular protein extracts were analyzed by Western blotting with an anti-IκBα antibody (top panel). The same membranes were stripped and reprobed with an anti-ß-actin antibody as a control for equal protein loading (bottom panel). Relative levels of IκBα in the cytoplasm were determined by densitometry and then normalized to ß-actin. Western blot data presented are representative of those obtained from 3 separate experiments.

NF-κB is known to be the transcription factor for numerous inflammatory mediators including proinflammatory cytokines. sTNF-α, a potent inflammatory cytokine, has been shown to induce IκB-α degradation and subsequently activate NF-κB leading to proinflammatory cytokine transactivation (15). To further clarify whether the effect of mTNF-α reverse signaling on cytokine expression was mediated through NF-κB transactivation, U937 cells were subjected to cytoplasmic protein isolation, and the levels of IκB-α in cytoplasm were analyzed by Western blotting. As seen in Fig. 3, sTNFRI alone did not alter the basal level of IκB in the cytoplasm compared to the untreated control, whereas both concentrations of sTNF-α (10 U/ml and 100 U/ml) reduced cytoplasmic IκB-α levels, with a better effect of sTNF-α at the dose of 100 U/ml (lane 5 vs. lane 3). As expected, sTNF-α-induced IκB-α degradation was increased by pre-activating mTNF-α reverse signaling with sTNFRI (lane 4 vs. lane 3 and lane 6 vs. lane 5), and the cytoplasmic levels of IκB-α were decreased by ≥20% (lane 6 vs. lane 5). These data suggest that the reverse signaling of mTNF-α enhanced sTNF-α-induced IκB-α degradation and NF-κB activation in U937 cells.

Enhancement of mRNA degradation of IL-1ß and IL-8 by reverse signaling through mTNF-α after sTNF-α-induced activation of U937 cells. Although sTNFR exists normally at low level in the circulation, it is increased rapidly and markedly during infection and inflammation. LPS-induced sTNF-α is transitory *in vivo* (22). Therefore, the effect of mTNF-α reverse signaling on the sTNF-α post-activated U937 cells was examined. For this, U937 cells were stimulated with 100 U/ml sTNF-α for 4 h, and then the cells were washed for 3 times to remove sTNF-α and treated either with culture medium or 10 μg/ml sTNFRI for different times. mRNA levels of proinflammatory cytokines were analyzed by RT-PCR. As observed in Fig. 4, the mRNA expression of IL-1ß and IL-8 was induced to a higher level following sTNF-α stimulation (lane 1), and the levels of the cytokine mRNA were gradually decreased after the sTNF-α depletion from the cultures. At 1 h IL-1ß mRNA and IL-8 mRNA levels were reduced to 60% and 43%, respectively. However, the reverse signal triggered by sTNFRI enhanced mRNA degradation of the proinflammatory cytokines after depletion of sTNF-α. At 1 h following sTNFRI treatment, mRNA levels of IL-1ß and IL-8 were reduced to 30% and 24% (lane 7), and at 2 h only 10% and 8% were left, respectively (lane 8). Thus, the reverse signaling of mTNF-α reduced mRNA stability of the cytokines, and the half-life of IL-1ß mRNA and IL-8 mRNA was decreased from 70 min and 60 min to 45 min and 35 min, respectively (Fig. 4).

Overexpression of a mutant mTNF-α lacking the intracellular segment in U937 cells increased mRNA stability of IL-1ß and IL-8 and impaired degradation of the cytokine mRNA. To verify the role of mTNF-α-mediated reverse signaling in modulation of mRNA stability of the inflammatory cytokines in this model system, a mutated form of mTNF-α lacking the cytoplasmic fragment was constructed and was introduced into U937 cells by transient transfection. The expression of wild-type (wt) mTNF-α and the mutant mTNF-α in U937 cells was determined by Western blot analysis. As seen in Fig. 5, the endogenous 26-kD mTNF-α was expressed in non-transfected

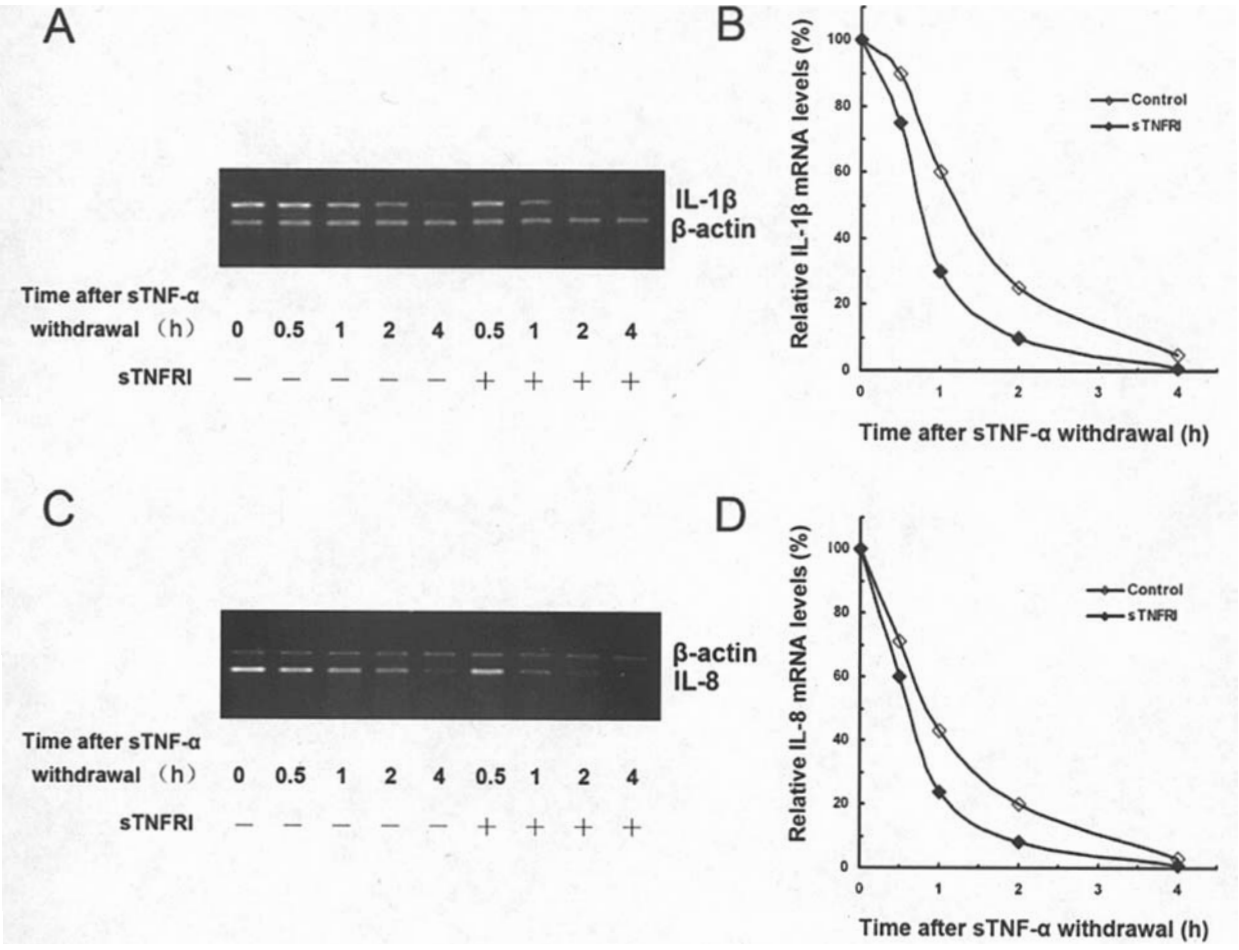

Figure 4. Enhancement of proinflammatory cytokine mRNA degradation in monocytic U937 cells mediated via mTNF-α reverse signaling. U937 cells ($1x10^6$/ml) were stimulated with 100 U/ml sTNF-α for 4 h, the cells were then washed 3 times to remove sTNF-α and treated either with culture medium or with 10 μg/ml sTNFRI for the indicated time points. Total cellular RNA was isolated, and the mRNA levels of proinflammatory cytokines (IL-1ß and IL-8) were analyzed by RT-PCR (panels A and C). Relative mRNA levels of IL-1ß or IL-8 were determined by densitometry and then normalized to ß-actin, and these data are shown in panels B and D. The results shown are representative of 3 independent experiments.

cells and in U937 cells transfected with the empty vectors. By contrast, a 26-kD band for endogenous mTNF-α and a 20-kD band for the mutant mTNF-α lacking the majority of the cytoplasmic segment were observed in the cells transfected with Δ-73~-47mTNF-α, while only a 26-kD band for mTNF-α showed up in the cells transfected with wt-TNF-α, and its level of expression was increased by 30%, as compared to that of the non-transfected cells, indicating that expressed protein includes both endogenous and transfected 26-kD mTNF-α.

Based on the results in Fig. 4, we chose 1-h post depletion of sTNF-α for determining whether overexpression of the mutant mTNF-α changed the mRNA levels of IL-1ß and IL-8 in sTNFRI-treated and -untreated U937 cells. As shown in Fig. 6, the treatment with sTNFRI increased the mRNA degradation of IL-1ß and IL-8 in non-transfected cells and in cells transfected with the empty vectors (lanes 5 and 6 vs. lanes 1 and 2 from left). In wt-mTNF-α-transfected cells overexpressing mTNF-α, sTNFRI treatment significantly reduced mRNA levels of both cytokines, as compared with those in sTNFRI untreated cells, resulting in ~90% degradation of the cytokine mRNA. However, in the cells overexpressing Δ-73~-47mTNF-α, the binding of sTNFRI to mTNF-α substantially increased mRNA levels of IL-1ß and IL-8, as compared to those in the non-transfected cells and in the cells transfected with the empty vectors or wt-mTNF-α (Fig. 6). These data suggest that the reverse signaling through mTNF-α is involved in the regulation of mRNA stability of the inflammatory cytokines induced by sTNF-α in human monocytic U937 cells.

Discussion

In the present study, we provided evidence demonstrating that stimulating mTNF-α with sTNFRI prior to sTNF-α activation elicited a positive regulatory signal that sensitized U937 monocytic cells to sTNF-α-induced responses, whereas treatment of sTNFRI following sTNF-α activation of the cells exerted a distinct negative signal that downregulated the mRNA levels of proinflammatory cytokines through the enhancement of cytokine mRNA degradation in this model system.

Since U937 cells express both mTNF-α and TNFR, and sTNF-α is from cleavage of mTNF-α, these cells may receive two signals simultaneously through the binding of sTNF-α or

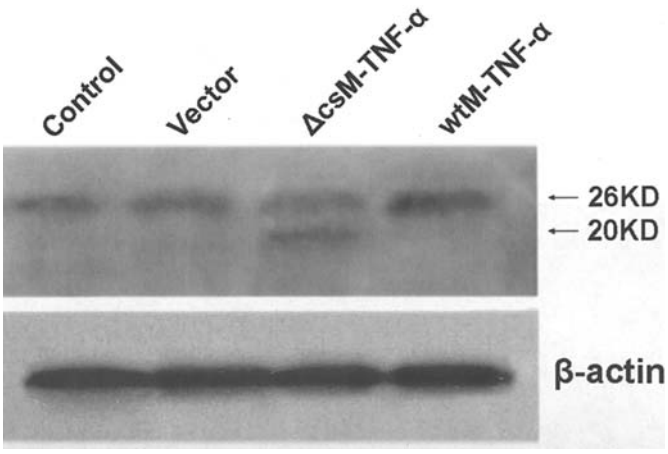

Figure 5. Demonstration of transient expression of ΔcsmTNF-α mutant in U937 human monocytic cells. U937 cells were transiently transfected with three forms of plasmid vectors by electroporation, respectively: a vector expressing a mutant mTNF-α (ΔcsmTNF-α) lacking the cytoplasmic segment from the position -73 to -47, a vector expressing wild-type TNF-α (wtTNF-α), and the empty vector pCDNA 3.0. The transfection efficiency was monitored by cotransfection of an expression vector containing the green fluorescent protein (pEGFP-N1). After a 48-h recovery, the cells were collected and lysed. The expression of 26-kD mTNF-α and 20-kD ΔcsmTNF-α mutant in U937 cells was analyzed by Western blotting with an anti-TNF-α monoclonal antibody. Western blot results shown are representative of 3 independent experiments.

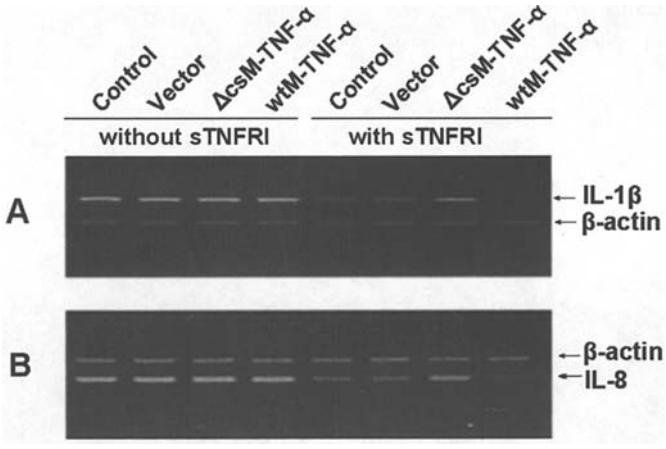

Figure 6. Induction of reverse signaling through mTNF-α after sTNF-α-induced activation of U937 cells leads to reduction in mRNA levels of proinflammatory cytokines. Three types of plasmid vectors (the empty vector, ΔcsmTNF-α, and wtTNF-α) were transiently transfected into U937 cells by electroporation, respectively. The transfection efficiency was monitored by cotransfection of an expression vector containing the green fluorescent protein (pEGFP-N1). After 48 h of transfection, the transfected cells were stimulated with 100 U/ml sTNF-α for 4 h. The cells were then washed and treated with fresh culture medium (lanes 1-4 from left) or with fresh culture medium containing 10 μg/ml sTNFRI (lanes 5-8 from left) for 1 h. The mRNA levels of IL-1ß (panel A) and IL-8 (panel B) were analyzed by RT-PCR, as described in Materials and methods. The data presented are representative of 3 separate experiments.

mTNF-α to TNFR (forward signaling) and the binding of TNFR to mTNF-α (reverse signaling). Our studies (23) have demonstrated that sTNF-α alone was able to enhance phagocytosis, increase the mRNA accumulation of cytokines, such as TNF-α, IL-1ß and IL-8, and induce the expression of ICAM-1 and degradation of IκB-α in monocytic U937 cells. However, mTNF-α, as a single agent, failed to activate U937 cells in regard to these aspects, as compared to sTNF-α. These observations suggest that mTNF-α, by itself, does not serve as a proinflammatory player acting through the forward manner in the regulation of inflammation responses. This prompted us to investigate whether mTNF-α may exert an action in modulating immune-inflammatory responses through the reverse manner.

To address this issue, we chose the human monocytic cell line U937 as a model for studying the role of mTNF-α-mediated reverse signaling in regulating sTNF-α-induced activation of these cells because mTNF-α is expressed on 45% of U937 cells. sTNFRI was used as a ligand for inducing reverse signaling through mTNF-α in our experiments, and we treated the cells with sTNFRI prior to activation by sTNF-α and evaluated some parameters of U937 cell activation. Our results indicated that the pre-activated reverse signaling via mTNF-α sensitized U937 cells to sTNF-α and enhanced sTNF-α-induced activation of the cells. This conclusion is supported by several lines of evidence: i) Pretreatment of sTNFRI increased sTNF-α-induced reactive oxygen production in a dose-dependent manner; ii) Pretreatment of sTNFRI enhanced sTNF-α-induced mRNA levels of proinflammatory cytokines, such as IL-1ß, IL-8 and TNF-α itself; and iii) Pretreatment of sTNFRI augmented sTNF-α-induced IκB-α degradation which leads to NF-κB activation. NF-κB is believed to play an essential role in developing inflammation because it is the transcriptional activator of myriad inflammatory mediators including proinflammatory cytokines and chemokines. Full activation of monocytes/macrophages needs dual signals: one signal is from IFN-γ, and the other is from TNF-α or CD40L (19). The main function of the latter signal is to enhance the susceptibility of monocytes/macrophages to IFN-γ. We believe that the priming of U937 cells by sTNFRI may serve as a costimulatory signal for optimal activation of the cells by sTNF-α, and both signals act collaboratively or synergistically to activate U937 cells. This is consistent with a previous study showing that a synergistic role of mTNF-α reverse signaling and sTNF-α inflammatory action in inducing uninfected inflammation such as rheumatoid arthritis and chronic inflammatory arthritis in transgenic mice (24).

The mechanism underlying the synergistic action of sTNF-α and mTNF-α reverse signaling in the activation of U937 cells is unclear at this point. However, it is conceivable that the reverse signaling mediated by mTNF-α might augment TNF-α-induced responses through upregulating TNF and TNFR expression on the cells, as demonstrated in activated lymphocytes in our laboratory (data not shown). The more TNF-α and TNFR expression on the cells, the more activating signals the cells may receive. Additionally, the signal peptide of TNF-α, a 10-kDa fragment containing the putative nuclear localization signal, is able to translocate into the nucleus after cleavage from mTNF-α, which increases the expression of cytokines such as IL-1ß (25). Another possibility is that mTNF-α reverse signaling may reduce the threshold for cell activation in response to sTNF-α and thus make the cells more susceptible to sTNF-α-induced NF-κB activation and NF-κB responses. Normally, NF-κB remains sequestered in cytoplasm by binding to IκB protein which prevents NF-κB activation and

translocation into the nucleus. In the current study, the priming of U937 cells by TNFRI increased TNF-α-induced IκB-α degradation as compared to the cells treated with TNF-α alone. Thus, more NF-κB molecules were released and migrated into the nucleus for activating cytokine transcription and inducing cytokine production in this system. Moreover, it is possible that the pretreatment of the cells with TNFRI may induce or activate a transcriptional coactivator (or a transcriptional coregulator) which is required for the full transactivation of the inflammatory cytokine genes in response to TNF-α action. Nevertheless, the molecular basis for the reverse signaling through mTNF-α in modulating TNF-α-induced activation of U937 cells remains to be experimentally determined.

In contrast to our results, Eissner and colleagues reported (16) that the pre-activated reverse signaling via mTNF-α conferred the resistance of monocytes/macrophages to LPS stimulation, as shown by a decrease in TNF-α, IL-1 and IL-6 release. Although the mechanisms for these phenomena are not understood at present, this difference could be due to different cell types and different stimuli used in the two studies. We observed in this investigation that the effect of mTNF-α reverse signaling on U937 cell activation post sTNF-α stimulation differed from that of pre-activated reverse signaling. Instead of upregulating cytokine mRNA levels, we found that mTNF-α reverse signaling post-activation of the cells enhanced mRNA degradation of proinflammatory cytokines and led to rapid reduction in cytokine mRNA levels, such as IL-1ß and IL-8, in U937 cells. The enhancement of cytokine mRNA degradation by mTNF-α reverse signaling might be due to mRNA destabilization mediated through a p38 MAPK-dependent pathway. Waetzig and co-workers reported that mTNF-α reverse signaling induced TNF-α production and p38 activation in THP-1 human monocytic cells, and that infliximab-enhanced TNF-α gene expression was inhibited by the p38 inhibitor SB 203580 (26). The mRNAs regulated by p38 share a common A + U-rich element (ARE) in their 3'-untranslated regions (27). Rapid degradation of many labile mRNAs is regulated in part by the ARE. Therefore, mTNF-α reverse signaling may activate AUF1 through a p38-mediated pathway and destabilize IL-1ß mRNA and IL-8 mRNA via binding of the activated AUF1 to the ARE of the cytokines.

In an effort to better understand the mechanism for cytokine mRNA stability in U937 cells, we focused on the role of mTNF-α-mediated reverse signaling in this study. If the reverse signaling through mTNF-α is indeed responsible for the cytokine mRNA degradation in our model system, then over-expression of a dominant negative mTNF-α might prevent the cytokine mRNA from being degraded. To answer this question, we constructed an expression vector encoding an mTNF-α mutant. As phosphorylation of mTNF-α has been found to be restricted to serine residues within the N-terminal cytoplasmic portion of the molecule (28), and this site was identified as a substrate for casein kinase 1 (CK1). mTNF is dephosphorylated upon activation by sTNFR by presently unknown phosphatases (11), suggesting that the cytoplasmic domain of mTNF-α is critical for the biological actions of mTNF-α. Therefore, we assessed the role of reverse signaling via mTNF-α in cytokine mRNA stability by deleting the cytoplasmic segment -73~-47 including CK1 phosphorylation site to make an mTNF-α mutant that cannot transduce reverse signals. We transfected U937 cells with this vector so that the cells expressed both endogenous mTNF-α and the mTNF-α lacking the majority of the cytoplasmic fragment, and that the Δ-73~-47mTNF-α mutant could compete with endogenous mTNF-α for binding to sTNFRI but was unable to transduce reverse signals. Our results revealed that the mRNA degradation of IL-1ß and IL-8 was markedly reduced in cells expressing the mutant mTNF-α but not in cells expressing wild-type mTNF-α. In fact, the mRNA levels of the cytokines were moderately elevated in the U937 cells transfected with wild-type TNF-α than those in the non-transfected cells expressing only endogenous mTNF-α. These data suggest that mTNF-α reverse signaling is involved in the enhancement of mRNA degradation of IL-1ß and IL-8 after activation of the cells by sTNF-α.

In summary, we have shown in this study that pretreatment with sTNFRI, for inducing reverse signaling through mTNF-α, sensitized U937 cells to sTNF-α-induced activation, whereas binding of sTNFRI to mTNF-α after sTNF-α-induced activation of U937 cells reduced mRNA stability of IL-1ß and IL-8. Furthermore, cells overexpressing a wild-type mTNF-α were more susceptible to TNF-α-induced inflammatory response, while cells overexpressing a mutant mTNF-α failed to undergo cytokine mRNA degradation. These results suggest that mTNF-α-mediated reverse signaling may play opposite roles in modulating immune-inflammatory responses in U937 cells depending upon the status of the cells. It triggers a more vigorous inflammatory response when reverse signaling occurs prior to sTNF-α stimulation, while it is important for limiting inflammation reaction by terminating TNF-α-induced inflammatory responses when reverse signaling occurs after the sTNF-α signal. Thus, the present study points to the ability of mTNF-α-mediated reverse signaling to serve a dual role in the regulation of immune-inflammatory responses and control of inflammatory reactions in monocytes and macrophages. Investigations are in progress to unveil the signaling pathway(s) through which, such as serine phosphorylation (11,28), nuclear translocation (24) and palmitoylation (29), mTNF-α-mediated reverse signaling modulates immune responses and inflammatory reactions in human monocytic U937 cells and other immunocytes.

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