

# TRAF1 is a TNF inducible regulator of NF- $\kappa$ B activation

Isabelle Carpentier, Rudi Beyaert\*

Department of Molecular Biology, Flanders Interuniversity Institute for Biotechnology, University of Gent, K.L. Ledeganckstraat 35, B-9000 Gent, Belgium

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**Abstract** Tumor necrosis factor receptor (TNFR)-associated factor TRAF1 was first identified as a component of the TNFR2 signalling complex. Unlike the other members of the TRAF family, TRAF1 lacks the N-terminal ring finger motif and has a tissue specific expression. Here we demonstrate that expression of TRAF1 is induced by TNF and the protein kinase C (PKC) activator PMA, but not by interleukin-1 (IL-1). TNF-induced upregulation of TRAF1 could be prevented by pretreatment of the cells with the proteasome inhibitor MG-132, whereas the PKC inhibitor Ro31-8220 was without effect. Interestingly, overexpression of TRAF1 in HEK293T completely prevented NF- $\kappa$ B activation induced by TNF, IL-1, or overexpression of TRAF2 or TRAF6. These data suggest that inducible expression of TRAF1 may serve a negative regulatory function in NF- $\kappa$ B signalling pathways.

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**Key words:** TNF receptor factor;  
Nuclear factor  $\kappa$ B inhibition; Tumor necrosis factor

## 1. Introduction

Tumor necrosis factor (TNF) is a proinflammatory cytokine that plays a key role in the generation of local and systemic responses to infection, injury, and immunological changes. TNF exerts its effects through type 1 (TNFR1) or type 2 (TNFR2) receptors, which belong to a large TNFR superfamily that includes, among others, Fas (CD95), Wsl-1/DR3, CD30, CD40, Ox-40, and the lymphotoxin- $\beta$  receptor [1]. The identification of several proteins that bind directly or indirectly with the cytoplasmic domain of TNFR1 or TNFR2 has led to a better understanding of TNF signalling. Binding of TNF to TNFR1 induces the recruitment of the signalling protein TNFR1 associated death domain protein (TRADD [2]), which serves as a platform to recruit at least three additional mediators: TNFR associated factor 2 (TRAF2 [3]), receptor interacting protein (RIP [4]), and Fas-associated protein with death domain (FADD [5]). TNFR2 triggering results in direct recruitment of TRAF2 and its relative TRAF1 [6]. To date, the TRAF family consists of six members that are involved in intracellular signal transduction by several mem-

bers of the TNFR and interleukin-1 (IL-1) receptor superfamily, including TNFR1, TNFR2, CD30, CD40, IL-1 receptor and Toll-like receptor-4 (reviewed in [7]). Transfection experiments implicated TRAF2 as a critical mediator of nuclear factor  $\kappa$ B (NF- $\kappa$ B [8]), c-jun N-terminal kinase (JNK [9]) and p38 mitogen activated protein kinase (MAPK [10,11]) activation by TNF. However, TRAF2 negative cells derived from TRAF2 deficient mice are severely defective in TNF induced JNK activation, whereas they still exhibit considerable, albeit slower, NF- $\kappa$ B activation [12]. The basis for this discrepancy is not understood, but it has been suggested that TRAF5 may substitute for TRAF2 in NF- $\kappa$ B activation [12]. TRAF6 is recruited to the IL-1 receptor and is crucial for IL-1 induced activation of NF- $\kappa$ B, JNK and p38 MAPK [13,14]. So far the functions of TRAF1, 3 and 4 remain largely unknown.

All TRAFs are composed of a highly conserved C-terminal TRAF domain which mediates homotypic and heterotypic TRAF-TRAF interactions, as well as the interaction with other signalling proteins. In the case of TRAF2, these include TRADD, TNFR2, RIP and the NF- $\kappa$ B inducing kinase NIK (reviewed in [7]). A more variable N-terminal domain, which contains a ring finger and several zinc finger motifs, is also found in all TRAFs with the exception of TRAF1. Although this N-terminal domain has been shown previously to be required for NF- $\kappa$ B activation [8,13,15], the underlying mechanism is still unclear. In this report, we have focused on the function of TRAF1, which differs from the other TRAFs not only at the structural level, but also by its tissue specific expression [6]. We demonstrate that TRAF1 expression is highly inducible by TNF and phorbol myristate acetate (PMA). Moreover, overexpression of TRAF1 inhibits NF- $\kappa$ B activation induced by TNF and IL-1, suggesting that TNF induced expression of TRAF1 provides a negative regulatory signal in NF- $\kappa$ B signalling pathways.

## 2. Materials and methods

### 2.1. Cells and reagents

The HeLa cell line was a gift from Dr. H. Bujard (ZMBH, Heidelberg). HEK293T cells were a gift from Dr. M. Hall (Department of Biochemistry, University of Birmingham). U937 cells were obtained from Biogent (Belgium). PC60 cells stably expressing human TNFR1 and TNFR2 have been described previously [16]. HeLa and HEK293T cells were cultured in Dulbecco's modified Eagle's medium, U937 and PC60 cells were cultured in RPMI 1640. Media were supplemented with 2 mM glutamine, 10% fetal calf serum,  $10^6$  U/l streptomycin and 100 mg/l penicillin. 0.4 mM sodium pyruvate or 100  $\mu$ M  $\beta$ -mercaptoethanol were also added in the case of HEK293T or PC60, respectively. MG-132 and Ro31-8220 were purchased from Calbiochem (La Jolla, CA), SB203580 was from Alexis Biochemicals (San Diego, CA), PMA was from Sigma (St. Louis, MO). Immobilized protein A on trisacryl was purchased from Pierce Chemical Co. (Rockford, IL).

\*Corresponding author. Fax: (32) (9) 264.53.48.  
E-mail: rudi.beyaert@dmb.rug.ac.be

**Abbreviations:** FADD, Fas-associated protein with death domain; IL-1, interleukin-1; MAPK, mitogen activated protein kinase; NF- $\kappa$ B, nuclear factor  $\kappa$ B; PKC, protein kinase C; PMA, phorbol myristate acetate; RIP, receptor interacting protein; TNF, tumor necrosis factor; TNFR, TNF receptor; TRADD, TNFR1-associated death domain protein; TRAF, TNFR associated factor

## 2.2. Plasmids and antibodies

pRK5-TRAF1, pRK5-TRAF2, pRK5-TRAF6 were a generous gift from Dr. D.V. Goeddel (Tularik, San Francisco, CA). pNFconluc, which carries a luciferase gene under the control of a minimal promoter preceded by three NF- $\kappa$ B binding sites, was provided by Dr. A. Israel (Institut Pasteur, Paris). pUT651, a  $\beta$ -galactosidase expression plasmid, was from Eurogentec (Seraing). pCDNA1-RIP<sub>E</sub> and pCDNA1-TRADD<sub>E</sub> were prepared by cloning the PCR fragment encoding RIP and TRADD in the pCDNA1 vector in frame with a C-terminal E-tag. Anti-TRAF1 (H-132) and anti-TRAF2 (C-20) antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA); anti-E-tag antibodies were from Pharmacia Biotech (Uppsala).

## 2.3. Biological IL-6 assay

Secreted IL-6 was quantitated according to its growth stimulatory effect on 7TD1 cells [17]. Cell proliferation was assessed by colorimetric determination of hexosaminidase levels [18].

## 2.4. NF- $\kappa$ B dependent reporter gene assay

HEK293T cells ( $3 \times 10^5$ ) were seeded in six well plates. The next day cells were transfected by the calcium phosphate precipitation method with 150 ng pUT651, 150 ng pNFconluc, 750 ng pRK5-TRAF1 and 250 ng empty vector, pRK5-TRAF2 or pRK5-TRAF6. Twenty hours later, cells were trypsinized and one tenth of the cells coming from a six well plate were transferred to a 24 well plate. One day later, cells were either left untreated or treated for 6 h with 1000 IU/ml TNF, 7000 IU/ml IL-1 or 200 ng/ml PMA. Cell lysates were prepared and NF- $\kappa$ B dependent luciferase (luc) gene expression was measured and normalized for  $\beta$ -galactosidase (gal) activity as described previously [19].

## 2.5. Western blot analysis of TRAF1 expression

Cell extracts were prepared in luciferase lysis buffer containing 25 mM Tris-phosphate pH 7.8, 2 mM DTT, 2 mM CDTA, 10% glycerol, 1% Triton X-100, 1 mM PMSF, 0.1 mM aprotinin and 1 mM leupeptin. Insoluble material was removed by centrifugation in an Eppendorf centrifuge. Samples containing equal amounts of protein were separated on 10% SDS-polyacrylamide gels and blotted onto nitrocellulose. Blots were blocked for 2 h in phosphate buffered saline supplemented with 3% non-fat dry milk and 0.2% Tween-20. Blots were probed with a 1:500 dilution of anti-TRAF1 antibodies in blocking buffer, followed by a 1:2000 dilution of donkey horseradish peroxidase coupled anti-rabbit immunoglobulin. Bands were visualized with enhanced chemiluminescence according to the protocol of the manufacturer (Amersham Life Sciences, Amersham).

## 2.6. Coimmunoprecipitation

HEK293T cells ( $1.5 \times 10^6$ ) were seeded in 9 cm petri dishes. The next day cells were transfected via the calcium phosphate precipitation method with 1  $\mu$ g pRK5-TRAF2 and 1  $\mu$ g pCDNA1-TRADD<sub>E</sub> or pCDNA1-RIP<sub>E</sub>, in the absence or presence of 3  $\mu$ g pRK5-TRAF1. Empty vector was added to a total amount of 5  $\mu$ g DNA. Twenty-four hours after transfection, cells were lysed in 20 mM Tris-HCl pH 7.5, 150 mM NaCl, 1 mM EDTA, 1% Triton X-100, 1 mM PMSF, 0.1 mM aprotinin and 1 mM leupeptin, and lysates were incubated for 2 h with 2  $\mu$ g anti-E-tag antibody. Immune complexes were incubated for 1 h with protein A-trisacryl beads, which were subsequently washed four times with lysis buffer containing 700 mM NaCl. Coprecipitating proteins were separated by SDS-polyacrylamide gel electrophoresis and analyzed by Western blotting with a 1:1000 dilution of TRAF-2 (C-20) antibodies, followed by a 1:2000 dilution of donkey horseradish peroxidase coupled anti-rabbit immunoglobulin. Bands were visualized with enhanced chemiluminescence according to the protocol of the manufacturer (Amersham Life Sciences, Amersham).

## 3. Results

### 3.1. TRAF1 is inducible by TNF, but not by IL-1

Constitutive TRAF1 mRNA expression can only be detected in limited tissues such as spleen, lung and testis [6]. Similarly, we could not detect any TRAF1 expression in various cell lines, including HeLa, PC60, and U937. Therefore, we analyzed whether TRAF1 expression could be induced by

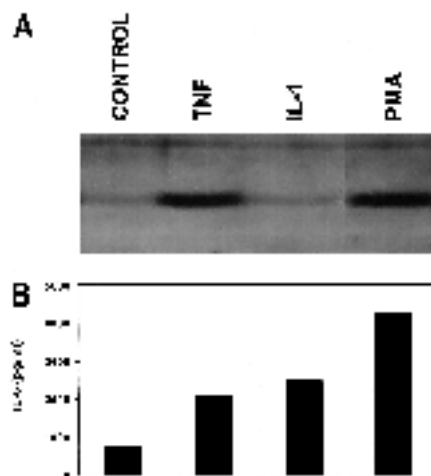


Fig. 1. TNF and PMA induce TRAF1 expression. A:  $2 \times 10^5$  HeLa cells were left untreated (control) or treated for 18 h with 1000 IU/ml TNF, 7000 IU/ml IL-1 or 200 ng/ml PMA. 200  $\mu$ g total cellular protein was analyzed by SDS-PAGE and immunoblot analysis with anti-TRAF1 antibodies. B: IL-6 levels in the supernatant of the cells that were treated as described in A were measured in an IL-6 bioassay. Results are representative for three independent experiments.

stimulation with TNF, IL-1 or the protein kinase C (PKC) activator PMA. HeLa cells were treated with these agents for 18 h and analyzed for TRAF1 expression by Western blot analysis with a TRAF1 specific antiserum. Whereas TRAF1 could not be detected in unstimulated cells, TRAF1 was well expressed in TNF and PMA stimulated HeLa cells (Fig. 1A). A similar upregulation of TRAF1 by TNF and PMA could be observed in U937 and PC60 cells (data not shown). Although TNF induced gene expression is mostly mimicked by IL-1, to our surprise the latter was not able to induce TRAF1 expression in HeLa cells. This differential regulation is not due to a general lack of IL-1 responsiveness of HeLa cells, because IL-1 was still able to induce IL-6 gene expression in these cells (Fig. 1B). Time kinetics revealed that the upregulation of TRAF1 protein expression is detectable 4 h after the start of TNF treatment, and lasts for at least up to 72 h (Fig. 2). In contrast to TRAF1, the expression of other TNFR associated proteins such as TRAF2, FADD, TRADD or RIP was not modulated by TNF treatment (data not shown).

### 3.2. Signalling pathways involved in TRAF1 induction

TNF and PMA are both known activators of PKC [20,21]. Therefore, we investigated whether the upregulation of TRAF1 by these stimuli was mediated by PKC. For this purpose, we pretreated HeLa cells for 2 h with the PKC inhibitor Ro31-8220, and then stimulated the cells with TNF or PMA for 18 h. Western blot analysis revealed that TRAF1 induction by PMA can be considerably inhibited by 0.5  $\mu$ M Ro31-8220 (Fig. 3A). In contrast, TNF mediated induction of TRAF1 was unaffected by Ro31-8220. These results suggest that in the case of TNF, TRAF1 induction is PKC independent. As we previously already demonstrated a role for p38 MAPK in TNF induced expression of IL-6 and granulocyte/macrophage colony stimulating factor [19], we also analyzed the role of this kinase in TRAF1 expression by pretreating HeLa cells with the p38 MAPK inhibitor SB203580. However, the latter did not affect the TNF induced expression of

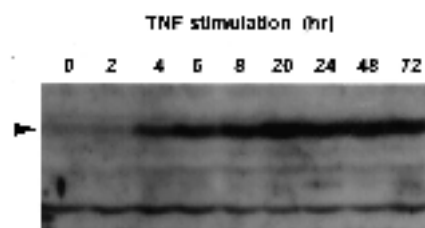


Fig. 2. Time kinetics of TRAF1 expression induced by TNF.  $2 \times 10^5$  HeLa cells were treated with 1000 IU/ml TNF for the indicated times. Cells were harvested and 200  $\mu$ g total cellular protein was analyzed by SDS-PAGE and immunoblot analysis with anti-TRAF1 antibodies. The arrowhead indicates the TRAF1 protein.

TRAF1 (data not shown), indicating that also p38 MAPK is not involved in the upregulation of TRAF1 by TNF.

Because TNF is known to be a very potent activator of NF- $\kappa$ B, we subsequently analyzed the role of this transcription factor in TRAF1 expression by pretreating HeLa cells for 2 h with the proteasome inhibitor MG-132. The latter has previously been shown to inhibit NF- $\kappa$ B activation by preventing the degradation of the NF- $\kappa$ B inhibitory protein I $\kappa$ B [22]. 20  $\mu$ M MG-132 completely prevented the induction of TRAF1 by TNF (Fig. 3B). A similar effect could be obtained with the NF- $\kappa$ B inhibitors BAY11-7085 and gliotoxin ([23,24]; data not shown). These results suggest a role for TNF induced NF- $\kappa$ B activation in the upregulation of TRAF1.

### 3.3. TRAF1 inhibits NF- $\kappa$ B dependent gene expression

TRAF1 differs from the other members of the TRAF family by the lack of an N-terminal ring finger structure. This domain was previously shown to be required for the NF- $\kappa$ B activating potential of TRAF2 and TRAF6 in the TNF initiated or IL-1 initiated signalling pathway, respectively [8,13,15]. Moreover, mutants of the latter proteins lacking the N-terminal ring finger structure can act as dominant neg-

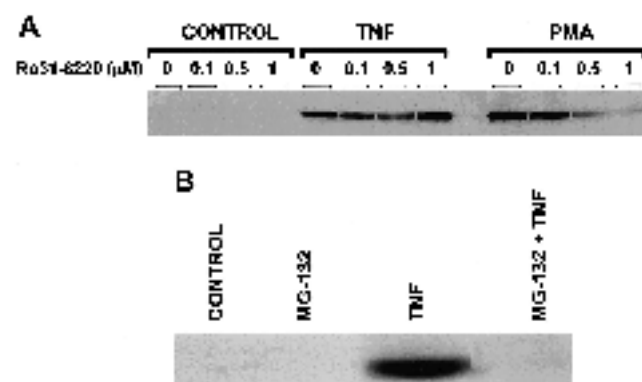


Fig. 3. Effect of PKC and proteasome inhibitors on induction of TRAF1. A:  $3 \times 10^5$  HeLa cells were left untreated (control) or pretreated for 2 h with the indicated concentrations of the PKC inhibitor Ro31-8220, and subsequently stimulated with 1000 IU/ml TNF or 200 ng/ml PMA. Cells were lysed 18 h later and 200  $\mu$ g total cellular protein was analyzed by SDS-PAGE and immunoblot analysis with anti-TRAF1 antibodies. B:  $3 \times 10^5$  HeLa cells were left untreated (control) or treated for 2 h with 20  $\mu$ M of the proteasome inhibitor MG-132 prior to treatment with 1000 IU/ml TNF. Cells were lysed 18 h later and 200  $\mu$ g total cellular protein was analyzed by SDS-PAGE and immunoblot analysis with anti-TRAF1 antibodies.

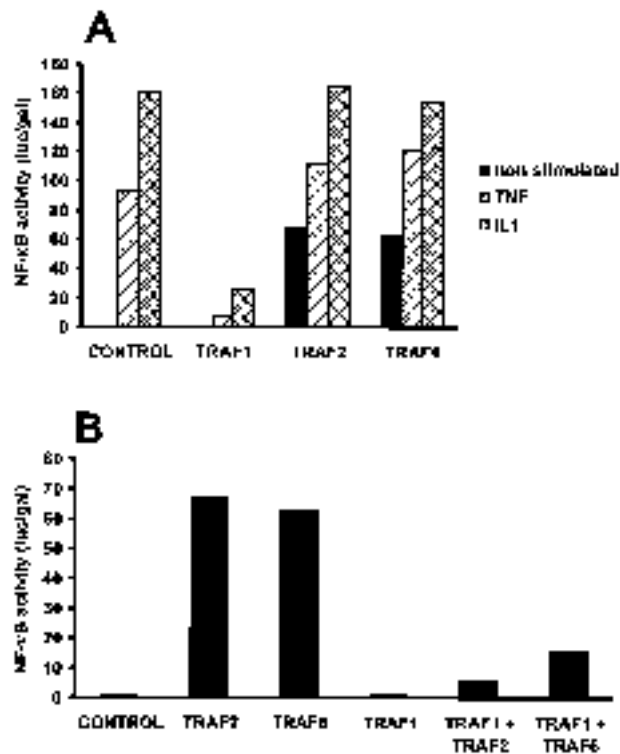


Fig. 4. Overexpression of TRAF1 inhibits NF- $\kappa$ B activation. A: HEK293T cells transiently transfected with a NF- $\kappa$ B luciferase (luc) reporter construct and a  $\beta$ -galactosidase (gal) expression vector were stimulated for 6 h with TNF and IL-1 or left untreated, and subsequently luciferase activity was determined and normalized for  $\beta$ -galactosidase activity. B: HEK293T cells were transiently transfected with a NF- $\kappa$ B luciferase reporter construct, a  $\beta$ -galactosidase expression vector, and specific TRAF expression vectors as indicated. Cells were analyzed 44 h later for luciferase and  $\beta$ -galactosidase activity. Results shown are representative for three independent experiments.

ative inhibitors of NF- $\kappa$ B activation [3,13]. Therefore, we analyzed whether overexpression of TRAF1 in HEK293T cells was able to prevent TNF or IL-1 induced NF- $\kappa$ B activation. Indeed, TRAF1 considerably decreased TNF and IL-1 induced expression of a NF- $\kappa$ B dependent reporter gene (Fig. 4A). In contrast, overexpression of TRAF2 or TRAF6 did not modulate the effect of TNF or IL-1, but already activated NF- $\kappa$ B as such. The latter can be explained by spontaneous oligomerization of TRAF2 or TRAF6 upon overexpression, which will also initiate signalling [15]. Coexpression of TRAF1 also prevented NF- $\kappa$ B activation induced by TRAF2 or TRAF6 (Fig. 4B), suggesting that TRAF1 interferes with TRAF mediated signalling.

### 3.4. TRAF1 overexpression has no effect on the binding of TRAF2 with TRADD or RIP

TRAF2 physically interacts with the TNFR adapter proteins TRADD and RIP, thereby linking the TNFR to the NF- $\kappa$ B activation pathway. TRAF1 also interacts with TRADD and RIP [3,4], but is unable to activate NF- $\kappa$ B. Moreover, TRAF1 is able to form heterodimers with TRAF2 [6]. It is therefore tempting to speculate that TRAF1 may sequester TRAF2 and thereby negatively regulate NF- $\kappa$ B activation. We therefore investigated whether overexpression of TRAF1 in HEK293T cells could prevent the TRAF2-RIP or TRAF2-

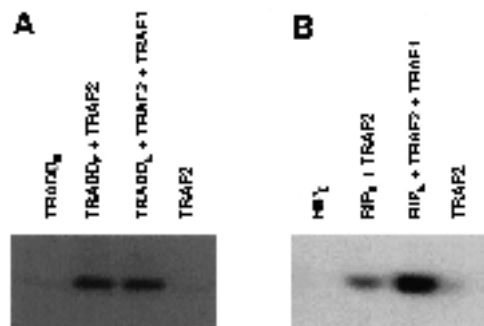


Fig. 5. TRAF1 has no effect on the coimmunoprecipitation of TRAF2 with RIP or TRADD. HEK293T cells were transiently transfected with expression plasmids for TRAF1, TRAF2, E-tagged TRADD (TRADD<sub>E</sub>) and/or E-tagged RIP (RIP<sub>E</sub>) as indicated. The next day, TRADD<sub>E</sub> (A) and RIP<sub>E</sub> (B) were immunoprecipitated with anti-E-tag antibodies and coimmunoprecipitation of TRAF2 was detected via Western blot analysis with anti-TRAF2 antibodies.

TRADD interaction. However, TRAF1 coexpression had no effect on the coimmunoprecipitation of TRAF2 with RIP or TRADD, respectively (Fig. 5). Although we cannot exclude an effect of TRAF1 on other protein interactions with TRAF2, the above results make it unlikely that TRAF1 interferes with NF- $\kappa$ B activation by competing with TRAF2 for binding to TRADD or RIP.

#### 4. Discussion

This study documents TRAF1 expression in response to TNF, IL-1 or PMA, and describes the biological function of TRAF1 in NF- $\kappa$ B signalling. We focussed our work on TRAF1 because Northern blot analysis has previously demonstrated that, unlike the other TRAF family members, TRAF1 expression is restricted to spleen, lung and testis [6]. In addition, TRAF1 differs from the other TRAFs by the lack of an N-terminal ring finger domain. Deletion of this domain in other TRAFs leads to the generation of dominant negative TRAF mutants, suggesting that the ring finger domain is critical for TRAF mediated signalling. Moreover, a similar dominant negative effect has been demonstrated for a splice variant of TRAF2, which contains an insert of seven amino acids in the ring finger structure [25], further demonstrating the importance of the RING finger in TRAF mediated signalling. Although TRAF1 was identified as a protein that is recruited to the TNFR2 in a heterodimeric complex with TRAF2 [6], its biological role has remained largely unclear. TRAF1 seems to be required for the recruitment of cellular inhibitor of apoptosis proteins and the zinc finger protein A20 to TNFR2 [26,27]. Finally, transgenic TRAF1 expression was shown to inhibit antigen induced cell death of CD8<sup>+</sup> T lymphocytes [28], as well as TNF induced apoptosis [29].

Our results demonstrate that overexpression of TRAF1 completely prevents TNF and IL-1 induced NF- $\kappa$ B activation in HEK293T cells. Moreover, we also show that TRAF1 expression can be induced by stimulation of cells with TNF or PMA. All together, these results suggest that induction of TRAF1 functions to downregulate NF- $\kappa$ B activation in response to TNF or IL-1. While this article was under revision, it was shown in an independent study that TRAF1 overexpression can induce a two-fold increase of TNF induced NF-

$\kappa$ B activation in HeLa cells [30]. At this moment, the reason for these contrasting results is still unclear. However, it should be mentioned that in the other study, transfections were performed with rather high concentrations of a TRAF1 expression plasmid (10  $\mu$ g versus 750 ng in our study). Alternatively, cell type dependent effects might be involved. In this context, we were also unable to detect an effect of IL-1 on TRAF1 expression in HeLa or PC60 cells, whereas IL-1 was shown in the other study to increase TRAF1 expression in SV80 cells [30] (although this was only demonstrated at the mRNA level). Similarly, we and others were unable to detect an effect of TNF on TRAF2 expression in several cell lines [30], whereas Wang and coworkers previously reported a TNF induced up-regulation of TRAF2 in HT1080 fibrosarcoma cells [29].

The TRAF1 gene was recently mapped and shown to contain binding sites for AP-1, CREB/ATF, and NF- $\kappa$ B [31]. Transfection of cells with an I $\kappa$ B- $\alpha$  super-repressor of NF- $\kappa$ B [19], as well as mutation analysis of TRAF1 gene promoter luciferase constructs [30], has shown the functional importance of the NF- $\kappa$ B elements. Our observations with chemical inhibitors of the NF- $\kappa$ B activation pathway further support the role of NF- $\kappa$ B in TRAF1 expression. However, because TNF as well as IL-1 activate NF- $\kappa$ B in several cell lines, including HeLa cells (our own unpublished observations), the differential effect of both cytokines on TRAF1 expression in HeLa cells cannot be explained by a difference in NF- $\kappa$ B activation. Further studies will be required to point this out in more detail.

The mechanism by which TRAF1 interferes with TNF and IL-1 induced NF- $\kappa$ B activation is still unclear. Because TRAF1 can bind via its C-terminal TRAF domain with several signalling molecules that are involved in NF- $\kappa$ B activation by TNF and IL-1 [3,4,32], it might prevent the interaction of these signalling molecules with upstream or downstream effector molecules of the NF- $\kappa$ B pathway. However, we were unable to demonstrate any effect of TRAF1 overexpression on the binding of TRAF2 with either TRADD or RIP, making the former hypothesis rather unlikely. Recently, TRAF1 has been shown to stabilize the expression of TRAF2A, which is a splice variant of TRAF2 that has been characterized as a natural inhibitor of TRAF2 mediated NF- $\kappa$ B activation [25]. Although the above observation can explain the effect of TRAF1 on TNF induced NF- $\kappa$ B activation, it fails to explain the inhibitory effect of TRAF1 on IL-1 induced NF- $\kappa$ B activation because the latter has been shown to be TRAF2A insensitive. A third and most likely explanation is that TRAF1 is responsible for the recruitment of cellular inhibitors of NF- $\kappa$ B activation to the TNF or IL-1 receptor complex. In this context, the zinc finger protein A20 has previously been shown to associate specifically with TRAF1-TRAF2 heterodimers [27], and to prevent NF- $\kappa$ B activation at the level of TRAF2 [33]. Experiments to verify whether TRAF1 recruits A20 or other proteins into the TNFR complex are under way.

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