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TNF receptor-associated factor 1 is a positive regulator of the NF-κB alternative pathway

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ABSTRACT

Tumor necrosis factor receptor-associated factor 1 (TRAF1) is unique among the members of the TRAF family, as it lacks the N-terminal RING/zinc-finger domain. Also the function of TRAF1 is not clearly established, with many papers reporting contradictory results. Here we show that TRAF1 interacts with BAFF receptor, a member of the TNF receptor family, and positively regulates activation of the alternative NF-κB pathway. Ectopic expression of TRAF1 causes degradation of TRAF3, stabilization of NIK, and processing of p100 to produce the mature form p52. In addition, we show that knocking-down expression of TRAF1 in the Hodgkin's disease derived cell line L1236, interfere with p100 processing and with p52 mediate gene transcription. Collectively these results support a role for TRAF1 as a positive regulator of the NF-κB alternative pathway.

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1. Introduction

The NF-κB family of transcription factors controls many biological processes, including inflammation, immunity and development of the secondary lymphoid organs (Vallabhapurapu and Karin, 2009). There are two distinct NF-κB activation pathways, termed the canonical and the alternative (a.k.a. noncanonical) NF-κB pathways. Briefly, the canonical NF-κB pathway, that is activated by inflammatory cytokines and stress pathways, leads to the degradation of the inhibitory subunit IκB, and to the nuclear accumulation of the RelA/p50 and c-Rel/p50 dimers (Vallabhapurapu and Karin, 2009). The alternative NF-κB pathway is activated by a selected group of members of the TNF receptor family such as CD40 and BAFF receptor (Vallabhapurapu and Karin, 2009). Activation of this pathway leads to the processing of p100 to produce p52, which forms heterodimer with RelB (Hacker and Karin, 2006; Scheidereit, 2006). A central component of this pathway is the protein kinase NIK that, in unstimulated cells, is suppressed by TRAF3, a well known interactor of BAFF receptor (Hauer et al., 2005), that induces NIK ubiquitination and degradation. Upon stimulation, TRAF3 undergoes signal-dependent degradation, mediated by TRAF2, c-IAP1 and c-IAP2, resulting in the accumulation and activation of NIK, and consequent processing of p100 and activation of the alternative pathway (Vallabhapurapu et al., 2008; Zarnegar et al., 2008).

Tumor necrosis factor (TNF) receptor-associated factor (TRAFs) is a family of adaptor proteins that are involved in signalling by the TNF receptor family, and toll/interleukin-1 receptor (TIR) family members (Hacker and Karin, 2006; Hayden et al., 2006). The TRAF proteins are characterized by the presence of a conserved carboxy-terminal homology domain of about 180 amino acids, the TRAF domain. This domain is involved in homo–heterodimerization with other members of the TRAF family, and interaction with many different proteins and receptors, thus acting as a scaffold protein organizing signalling complexes (Hacker and Karin, 2006; Hayden et al., 2006). Numerous studies demonstrated that the amino-terminal RING/zinc-finger domain of TRAFs is important for transmitting signals leading to the activation of NF-κB and JNK (Devin et al., 2000; Habelhah et al., 2004; Yamamoto et al., 2006; Bradley and Pober, 2001). Particularly, it has been demonstrated that the RING–finger domains of some TRAF proteins, such TRAF2 and TRAF6, have E3 ubiquitin ligase activity, which is required for their ability to transmit signals inside the cells (Lamothe et al., 2008). TRAF1, by contrast with other members of the family, does not have an N-terminal RING domain and does not have E3 ligase activity (Wajant et al., 2003). Contradictory results have been reported about its function. Some studies suggested that TRAF1 may function as a negative regulator of the CD40, IL1 and TNF-R signal transduction pathways (Tsitsikov et al., 2001). However, it has also been reported that TRAF1 enhances TNF-R2 induced NF-κB activation, thus acting as a positive regulator of TNF-signalling (Wicovsky et al., 2009). TRAF1 has been reported to protect CD8+ T cells from apoptosis (Sabbagh et al., 2006), and has been detected to be overexpressed in malignant lymphoid cells, suggesting a role for TRAF1 in sustaining lymphocyte proliferation (Izban et al., 2000). In order to study the role played by TRAF1 in lymphoid cells, we used TRAF1 as bait in a yeast two hybrid screening of a B cell library. We

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present evidence that TRAF1 interacts with the cytoplasmic domain of BAFF receptor and acts as a positive regulator of the alternative NF-κB pathway.

2. Experimental procedures

2.1. Cell culture and biological reagents

HEK293 cells were maintained in Dulbecco's modified Eagle's medium (Invitrogen) supplemented with 10% fetal bovine serum, 100 units/ml penicillin, 100 μg/ml streptomycin, and 1% glutamine. The Hodgkin's disease derived cell line L1236 (Wolf et al., 1996; Kanzler et al., 1996) was maintained in RPMI 1640 medium (Invitrogen) supplemented with 10% fetal bovine serum, 100 units/ml penicillin, 100 μg/ml streptomycin, and 1% glutamine.

Antibodies used for this study were: anti-FLAG (F7425, Sigma), anti-HA (SantaCruz 805), anti-TRAF1 (SantaCruz, 1830), anti-GST (G7781, Sigma), anti-p52 (SantaCruz 298), anti-actin (Santa Cruz Biotechnologies sc1616). Human BAFF (Peprotech Inc.) was used at 100 ng/ml.

Human TRAF1, Baff receptor, TRAF3, and p100/p52 was amplified by PCR from human PBL cDNA library (BD Clontech) and cloned into pcDNA3-FLAG, -HA for expression in mammalian cells, and pGEX2T vector for expression in *E. coli*. The human NF-κB inducing kinase (NIK) clone has been described (Leonardi et al., 2000). Deletion mutants were prepared by conventional PCR and cloned into pcDNA3-FLAG, -HA.

2.2. In vitro translation and GST pull-down assays

In vitro transcription and translation were carried out with 1 μg of FLAG-TRAF1 and FLAG-TRAF3 constructs according to the TNT Quick Coupled Transcription/Translation System protocol (Promega). GST pull-down assays were performed by incubating an aliquot of GST or GST-BAFF receptor bound to glutathione–sepharose beads (Amersham Biosciences) together with 10 μl of *in vitro* translated FLAG-TRAF1 and 10 μl of FLAG-TRAF3 protein in phosphate-buffered saline, 1% Triton X-100 buffer including Complete Protease Inhibitor mixture (Roche) for 2 h at 4 °C. Beads were then washed five times with the same buffer, resuspended in Laemmli buffer, and run on a SDS-polyacrylamide gel.

2.3. Transfection, immunoprecipitation, and luciferase assay

LipofectAMINE-mediated transfections were performed according to the manufacturer's instructions (Invitrogen). All transfections included supplemental empty vector to ensure that the total amount of transfected DNA was kept constant in each dish culture.

For immunoprecipitation of transfected proteins, HEK293 cells (3×10^6) were transiently transfected, and 24 h after transfection cells were lysed in Triton X-100 lysis buffer (20 mM Hepes pH 7.4, 150 mM NaCl, 10% glycerol, 1% Triton X-100, and Complete Protease Inhibitor mixture). After an additional 15 min on ice, cell extracts were centrifuged for 10 min at 14,000 × *g* at 4 °C, and supernatants were incubated for 3 h at 4 °C with anti-FLAG antibodies bound to agarose beads (M2, Sigma). The immunoprecipitates were washed five times with Triton X-100 lysis buffer and subjected to SDS-PAGE.

For luciferase assay, HEK293 cells (3×10^5) were seeded in 6-well plates. After 12 h cells were transfected with 0.5 μg of a reporter plasmid carrying the luciferase gene under the transcriptional control of the NF-κB alternative pathway consensus sequence (Bonizzi et al., 2004), and various combinations of expression plasmids. 24 h after transfection cell extracts were prepared and reporter gene activity was determined via the luciferase assay system (Promega). Expression of the pRSV-β-galactosidase vector (0.25 μg) was used to normalize transfection efficiency.

2.4. TRAF1 short hairpin RNA (shRNA)

To knockdown TRAF1 expression L1236 cells were transduced with the "SHVRS MISSION shRNA Lentiviral Particles" (Sigma). Four different lentiviral particles have been used (catalog #TRCN56883-6). After transduction, cells were selected with puromycin and TRAF1 expression was analyzed by Western blot.

2.5. Real Time PCR

The L1236 cells were stimulated with BAFF Ligand (Peprotech) for 4 h. Total RNA was extracted in accordance to standard methods. Real Time PCR was performed with SYBR Green PCR Master Mix kit (Applied Biosystem, Foster City, CA). The primers used for BAFF ligand are BAFF-Fw 5′-CGATGTATTCAAAATATGCCTGAAA-3′ and BAFF-Rev 5′-TGCAATGCCAGCTGAATAGC-3′. The cDNA concentrations were normalized by GAPDH mRNA content.

2.6. Statistics

Data were analyzed with ANOVA and a Student's *t*-test analysis. Data are presented as the means ± SD.*P* values <0.05 were considered significant.

3. Results

3.1. Identification of BAFF-R as a specific TRAF1 interacting protein

In order to study the role played by TRAF1 in lymphoid cells, we used TRAF1 as bait in a yeast two hybrid screening of a B cell cDNA library. Among 60 clones that were scored positive for interaction with the bait, 51 encoded the cytoplasmic portion of BAFF receptor. Interaction was confirmed in transfected cells. Expression plasmids encoding the HA-tagged cytoplasmic domain of BAFF-R and Flag-tagged TRAF1 were transfected into HEK293 cells. Coimmunoprecipitation experiments indicated that BAFF-R interacted with TRAF1 (Fig. 1A). Interaction was confirmed in a GST pull-down assay (Fig. 1B), in which a recombinant GST-BAFF receptor fusion was shown to interact with *in vitro* translated TRAF1. The interaction between BAFF receptor and TRAF3 was used as a positive control.

3.2. Domain mapping of the interaction between BAFF receptor and TRAF1

To determine the regions of TRAF1 required for interaction with BAFF-R, we constructed a series of deletion mutants of TRAF1 (Fig. 2A), and tested their ability to interact with the cytoplasmic domain of BAFF-R. Transient transfection of 293 cells and coimmunoprecipitation experiments showed that the amino acid residues between 300 and 350 of TRAF1 were required for interaction with BAFF-R (Fig. 2B).

3.3. TRAF1 positively regulates p100 processing

To investigate the functional consequence of the interaction between TRAF1 and BAFF-R, we investigated the role played by TRAF1 in modulating the activation of the NF-κB alternative pathway. To this purpose, we transfected NIK, TRAF3, and increasing levels of TRAF1 in HEK293 cells, and evaluated the processing of p100. As shown in Fig. 3A, NIK overexpression resulted in enhanced p100 processing, as indicated by the lower p100 levels and the

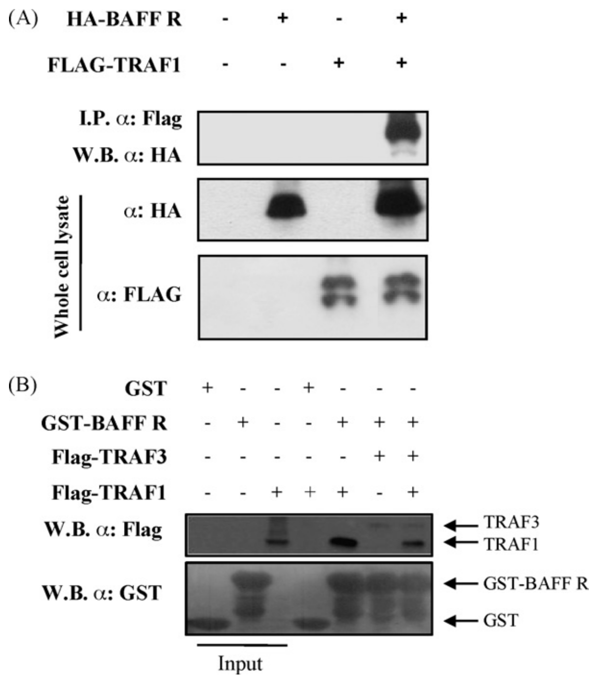

Fig. 1. BAFF-R interacts with TRAF1 in 293 cells. (A) 293 cells were transfected with expression plasmids for C-terminal HA-tagged BAFF-R and the indicated Flag-tagged TRAF1 protein. Cell lysates were immunoprecipitated with anti-Flag Ab. The immunoprecipitates were analyzed by Western blot with anti-HA (upper panel). Expression of BAFF-R and TRAF1 proteins was confirmed by Western blots with α-HA (middle panel) and α-Flag (lower panel) Abs, respectively. (B) GST pull-down assay. Purified GST or GST-BAFF receptor was incubated with *in vitro* translated FLAG-TRAF1 or FLAG-TRAF3, immunoprecipitated with Glutation Sepharose 4B, and analyzed by W.B. with α-Flag (upper panel) and with α-GST (lower panel).

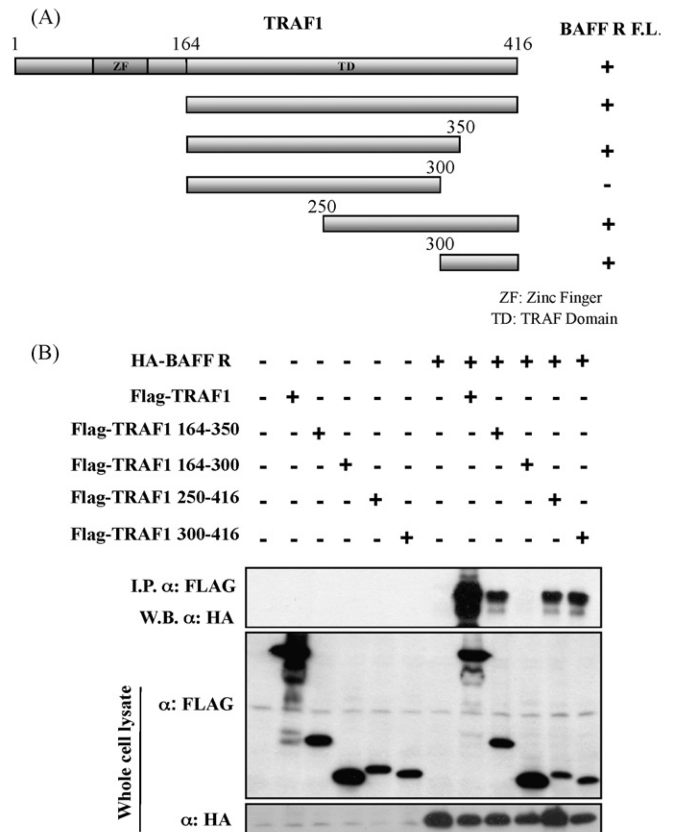

Fig. 2. Mapping of TRAF1 interaction with BAFF-R. (A) A schematic representation of the TRAF1 deletion mutants and their interaction with BAFF-R. TD, TRAF domain; ZF, zinc finger. (B) Coimmunoprecipitation between BAFF-R and TRAF1 mutants. 293 cells were transfected with expression plasmids for HA-tagged BAFF-R and the indicated C-terminal Flag-tagged TRAF1 mutants. Cell lysates were immunoprecipitated with α-Flag Ab and analyzed by Western blot with α-HA Ab (lower panel). Expression of TRAF1 mutants and BAFF-R was confirmed by Western blots with α-Flag (upper panel) and α-HA (middle panel) Abs, respectively.

increased level of the mature form p52. By contrast, in the presence of TRAF3, the level of NIK decreased and, as expected, the processing of p100–p52 was blocked. Ectopic expression of increasing levels of TRAF1, determined a decrease in the level of TRAF3, followed by stabilization of NIK, and increased p52 production.

We also evaluated the ability of TRAF1 to regulate the activity of an alternative NF-κB-driven reporter plasmid. HEK 293 cells were transfected with expression vectors encoding TRAF1, TRAF3, and sub-optimal concentration of NIK, and the activity of a cotransfected alternative NF-κB luciferase plasmid was evaluated. As shown in Fig. 3B, ectopic expression of TRAF1 was up-regulating the activity of the reporter plasmid induced by sub-optimal concentration of NIK. In addition, while TRAF3 was blocking the activity of the reporter plasmid induced by NIK, in the presence of TRAF1 this inhibition was removed.

3.4. *TRAF1 down-regulation decreases p100 processing*

To further demonstrate the positive role played by TRAF1 in regulating p100 processing, we knocked-down its expression in the Hodgkin's disease derived cell line L1236, by using shRNA. We utilized 4 different shRNA constructs named #1, #2, #3, #4, and 2 scrambled shRNA (ScrA and ScrB), as controls. As shown in Fig. 4A, all four shRNA decreased the expression of TRAF1, albeit at different level. We then evaluated the levels of p100 and p52 in these cells; knocking down TRAF1 expression caused a decrease in p100 processing. In order to evaluate if the decreased production of p52 was affecting transcription of genes controlled by the NF-κB alternative pathway, we evaluated by Real Time PCR, the mRNA level of Baff ligand, a known target of the NF-κB alternative pathway. As shown in Fig. 4B, while L1236 and control cells up-regulated transcription of Baff gene after stimulation, in cells knocked-down for TRAF1, the increased transcription of Baff gene was almost completely abrogated.

4. Discussion

In the present paper we identify TRAF1 as a BAFF receptor interacting protein, and present evidence for a role of TRAF1 as a positive regulator of the NF-κB alternative pathway.

In the current model of activation of the alternative pathway, TRAF3 constitutively binds to NIK and participates in its ubiquitination (Liao et al., 2004). After receptor engagement, TRAF3 is degraded, the level of NIK increases, and then NIK may phosphorylates IKKα to activate the complex (Liao et al., 2004). However, how engagement of the receptors that control B cell survival and development, such as CD40 and BAFF-R, regulates this process is currently unknown. We present evidence that the simple overexpression of TRAF1 decreases the level of TRAF3 and stabilizes NIK, thus mimicking receptor triggering. How does TRAF1 interfere with TRAF3 function? A possible explanation might be that TRAF1 compete with TRAF3 for binding to the receptor. However, our data suggest that this is not the case, as increasing amount of TRAF1 does not displace TRAF3 from binding to the receptor (data not shown). Another possibility is that TRAF1 displaces TRAF3 from NIK, thus preventing its ubiquitination and degradation. We are currently exploiting this possibility even if, at least in transfected cells, binding of TRAF3 to NIK is not affected by TRAF1 overexpression. There is another intriguing possibility: in the presence of

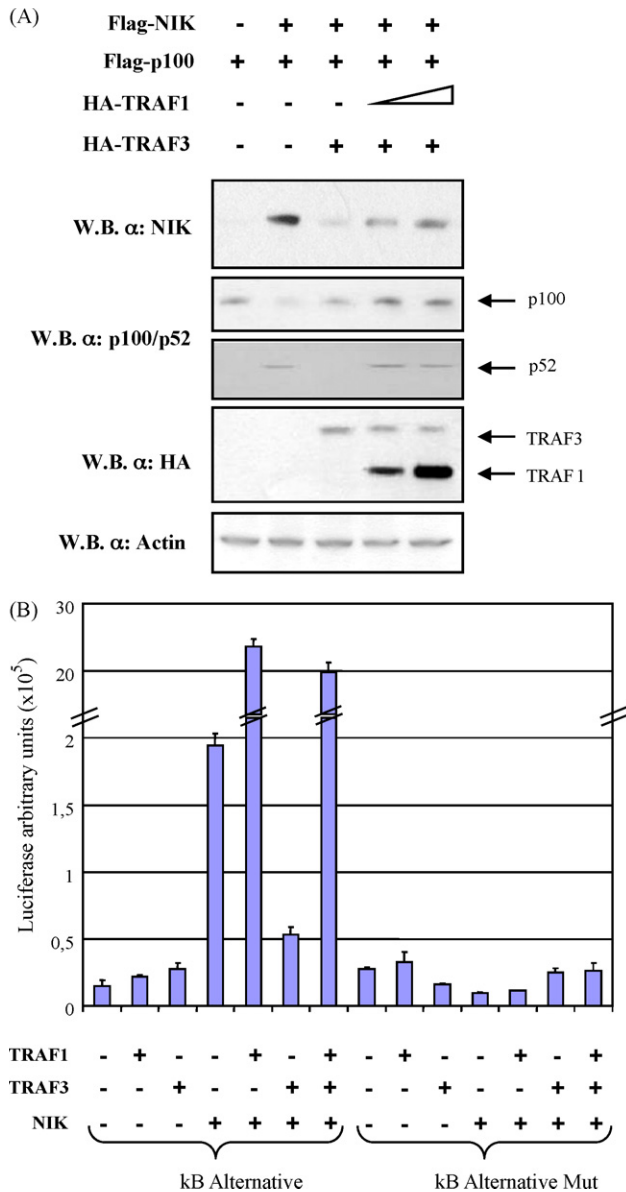

Fig. 3. TRAF1 positively regulates p100 processing. (A) 293 cells were transfected with expression plasmids for NIK, HA-TRAF1, HA-TRAF3 and FLAG-p100. Cell lysates were analyzed by Western blot with α-NIK (upper panel), α-HA and α-FLAG (middle panel) Abs. The Western blot anti-tubulin was used as a control for total proteins expression. (B) TRAF1 induces activation of the alternative pathway of NF-κB. HEK293 cells were transfected in triplicate with 0.5 μg the alternative-κB luciferase reporter together with NIK (0.01 μg), TRAF3 (0.1 μg), TRAF1 (0.5 μg). Data from a representative experiment, normalized for β-galactosidase activity, are shown as relative luciferase activity.

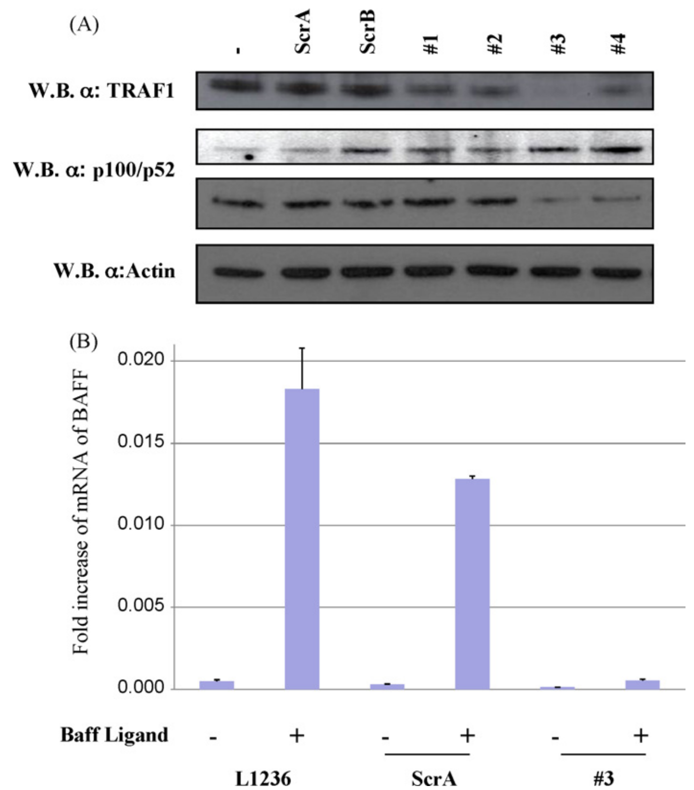

Fig. 4. The interference of TRAF1 decreases p100 processing and blocks the activation of BAFF pathway. (A) L1236 cells were infected with lentiviral particles carrying different TRAF1 interference constructs. The levels of TRAF1 (upper panel), p100 and p52 (middle panel) were analyzed by Western blot. The Western blot α-actin was used as a control for total proteins expression. (B) L1236, control, and interfered cells were stimulated with BAFF ligand (100 ng/ml) for 4 h and the level of Baff ligand mRNA was assessed by Real Time PCR. Data were analyzed with ANOVA and a Student's *t*-test analysis. Data are presented as the means ± SD. *P* values <0.05 were considered significant.

TRAF1, TRAF3 became substrate for another ubiquitin ligase activity, leading to TRAF3 degradation, and NIK stabilization. Indeed, it has been recently proposed by Karin and colleagues, and by Cheng and colleagues that TRAF3 degradation is mediated by a multimeric complex containg c-IAP1 and c-IAP2. This result suggests that TRAF1 may function to bridge interaction between IAPs and TRAF3. We are currently investigating if TRAF1 is part of this complex.

The present work also shows that blocking expression of TRAF1 by shRNA in Hodgkin's disease cells, blocks processing of p100. Elegant works recently found that a subgroup of multiple myeloma cells expresses elevated alternative pathway NF-κB activity, owing to amplification of NIK or mutation in TRAF3 (Annunziata et al., 2007; Keats et al., 2007). This result confirms the central role played by the alternative NF-κB pathway in lymphocyte survival, and suggests that amplification of TRAF1 may also be responsible for a subgroup of multiple myeloma. Indeed, TRAF1 mRNA and protein levels are typically increased in B cell malignancies, including non-Hodgkin lymphomas, chronic lymphocytic leukaemia, as well as the Reed-Stemberg cells of Hodgkin disease (Zapata et al., 2000). On the other hand, although T cells from TRAF1-null mice exhibited enhanced proliferation *in vitro*, their B cells have normal proliferation rates upon IgM or CD40 binding (Tsitsikov et al., 2001). In addition, TRAF1 transgenic mice that overexpress this protein in T cells have normal basal and CD3-stimulated proliferation compared to control littermates, suggesting that overproduction of TRAF1 does not alter CD3-mediated T cell proliferation. However, in the same experimental setting, the TCR-mediated apoptosis is reduced. How do we reconcile these disparate observations? It is possible that the difference in cell context may provide an explanation. However, it is also possible that *in vivo* the simple overexpression of TRAF1 is not sufficient to sustain proliferation and/or resistance to apoptosis in normal B cells.

Collectively, our results in addition to identify TRAF1 as a positive regulator of the alternative NF-κB activation pathway, identify TRAF1 as a potential target for treatment of myeloma.

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