# Opposing Roles for TRAF1 in the Alternative *versus* Classical NF-κB Pathway in T Cells[*]

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**Background:** The T cell costimulatory molecule 4-1BB signals through TRAF1 and TRAF2.
**Results:** TRAF1$^{-/-}$ T cells show hyperproliferation due to enhanced alternative NF-κB activation but have impaired classical NF-κB activation.
**Conclusion:** TRAF1 enhances 4-1BB induced classical NF-κB activation but restricts alternative NF-κB activation in the absence of costimulation.
**Significance:** This explains the contrasting roles attributed to TRAF1.

T cells lacking TRAF1 hyperproliferate in response to T cell receptor signaling but have impaired signaling downstream of specific TNFR family members such as 4-1BB. Here we resolve this paradox by showing that while TRAF1 is required for maximal activation of the classical NF-κB pathway downstream of 4-1BB in primary T cells, TRAF1 also restricts the constitutive activation of NIK in anti-CD3-activated T cells. Activation of the alternative NF-κB pathway is restricted in unstimulated cells by a cIAP1/2:TRAF2:TRAF3:NIK complex. Using knockdown of NIK by siRNA we show that in activated CD8 T cells TRAF1 is also involved in this process and that constitutive activation of the alternative NF-κB pathway is responsible for costimulation independent hyperproliferation and excess cytokine production in TRAF1-deficient CD8 T cells compared with WT CD8 T cells. The T cell costimulatory molecule 4-1BB critically regulates the survival of activated and memory CD8 T cells. We demonstrate that stimulation through 4-1BB induces cIAP1-dependent TRAF3 degradation and activation of the alternative NF-κB pathway. We also show that while both TRAF1 and cIAP1 have non-redundant roles in suppressing the alternative NF-κB pathway in T cells activated in the absence of costimulation, activation of the classical NF-κB pathway downstream of 4-1BB requires TRAF1, whereas cIAP1 plays a redundant role with cIAP2. Collectively these results demonstrate that TRAF1 plays a critical role in regulating T cell activation both through restricting the costimulation independent activation of NIK in activated T cells and by promoting the 4-1BB-induced classical NF-κB pathway.

Activation of T cells requires engagement of the T cell receptor, which confers specificity. However, for full activation, T cells require additional costimulatory signals that allow the cells to proliferate, survive, and produce cytokines (1). Although CD28 provides the primary costimulatory signal, the sustained survival of T cells requires that they receive additional signals through tumor necrosis factor receptor (TNFR)[3] family members (2, 3). For example, the TNFR family members 4-1BB, CD27, and glucocorticoid-inducible TNFR-related (GITR) have all been shown to play important roles in sustaining the survival of CD8 T cells following their initial antigen and CD28-dependent activation (3–8). This sequential regulation of T cell survival by different costimulatory receptors is thought to provide precise regulation of the duration of the T cell response to allow an appropriate response to pathogens, while preventing immune pathology (9).

Members of the TNFR family lack intrinsic enzymatic activity and instead rely on the recruitment of TNFR-associated factors (TRAFs) to induce their downstream signaling. TRAF proteins share a conserved C-terminal TRAF domain required for TRAF oligomerization and association with TNFR cytoplasmic tails. However, they differ in their N-terminal domains. TRAF2–6 contain a series of zinc finger domains and a RING finger domain that can potentially function as an E3 ubiquitin ligase to affect downstream signaling pathways (10). In contrast, TRAF1 differs from the other TRAF family members in lacking a RING finger domain and containing only one zinc finger, thus resembling a dominant negative form of TRAF2 (11).

In T cells, 4-1BB ligation results in the recruitment of TRAF1 and TRAF2 (12, 13). TRAF2 links 4-1BB to downstream activation of JNK(14), p38 (15), and NF-κB (13, 16), resulting in proliferation, cytokine production, and survival. In contrast, much less is known about the role of TRAF1. Our laboratory has shown that TRAF1 has an important role in mediating survival signaling downstream of 4-1BB, through induction of Bcl-x$_L$ and down-regulation of Bim (17). Consistent with this, Speiser *et al.* have shown that overexpression of TRAF1 in CD8 T cells prevents their death (18). In contrast, there have been conflict-

[3] The abbreviations used are: TNFR, tumor necrosis factor receptor; GITR, glucocorticoid-induced TNFR family related; TRAF, TNFR receptor-associated factor.

ing reports on the effect of TRAF1 deficiency on T cell responses. TRAF1$^{-/-}$ T cells were found to have increased responses to anti-CD3 stimulation as well as TNFR2 stimulation (19), but to have impaired survival during the expansion and memory phase of an immune response to influenza virus *in vivo* (20). Moreover, the role of TRAF1 in NF-κB activation in T cells is unclear. There are data supporting TRAF1's role as both a positive and negative regulator, possibly depending on the TNFR family member engaged and the cell type analyzed (21–24).

There are two types of NF-κB activation, the classical (or canonical) NF-κB pathway and the alternative (or non-canonical) NF-κB pathway (25). Activation of the classical NF-κB pathway downstream of TNFRs involves the recruitment of TRAF2 and the cIAP proteins, leading to the recruitment and ubiquitination of RIP1 and assembly of an IKK activating complex. This complex then leads to the phosphorylation and ubiquitin dependent degradation of the inhibitor IκB, releasing p65/p50 to translocate into the nucleus to activate transcription (25). Several TNFR family members have been shown to activate the alternative NF-κB pathway as well (26–31). Activation of the alternative NF-κB pathway involves the NF-κB inducing kinase (NIK)-dependent processing of NF-κB2 p100 into its active transcription regulatory fragment p52 (32). NIK activity is normally regulated by its constitutive degradation in unstimulated cells. The E3 complex responsible for its ubiquitination and subsequent degradation consists of cIAP1/2, TRAF2, and TRAF3 (33, 34). Upstream activating receptors of this pathway typically target either TRAF2 or TRAF3 for degradation to release NIK and allow its accumulation and activation inside cells (33–35). As well, smac mimetics, can lead to NIK accumulation through the redirection of cIAP1/2's E3 ligase activity toward self destruction (36, 37).

In this study we used TRAF1 deficient, as well as cIAP1 deficient, primary CD8 T cells combined with cIAP2 knockdown to explore the role of TRAF1 and cIAPs in NF-κB activation, specifically downstream of 4-1BB. We show that 4-1BB not only activates the classical NF-κB pathway, as previously reported (13, 16, 38), but also activates the alternative NF-κB pathway in primary T cells, as previously reported only in overexpression systems (31). We find that TRAF1$^{-/-}$ CD8 T cells as well as cells deficient in cIAP1 and cIAP2 have a defect in 4-1BB-induced classical NF-κB activation. In contrast, TRAF1$^{-/-}$ T cells hyperproliferate and have increased cytokine production in response to anti-CD3 stimulation alone compared with WT T cells due to costimulation-independent activation of the alternative NF-κB pathway. This opposing role for TRAF1 in enhancing the classical NF-κB pathway downstream of 4-1BB signaling but suppressing the alternative NF-κB pathway during initial TCR signaling explains the contrasting roles attributed to TRAF1 as a positive and negative regulator in T cells.

## EXPERIMENTAL PROCEDURES

*Mice*—C57BL/6 mice were obtained from Charles River Laboratories (Wilmington, MA). OT-I TCR transgenic mice have CD8 T cells specific for K$^b$ and OVA$_{257-264}$, SIINFEKL (39) and were originally obtained from the Jackson Laboratories and then crossed to generate CD45.1 OT-I WT and CD45.1 OT-I

TRAF1$^{-/-}$ mice as described (20). TRAF1$^{-/-}$ mice were originally provided by E. Tsitisikov (Center for Blood Research, Boston, MA) (19) but are now available from the Jackson Laboratories (Bar Harbor, ME). CIAP1$^{-/-}$ mice have been previously described (40) and had been backcrossed onto the C57BL/6 background for at least eight generations. These mice were crossed with the CD45.1 OT-I mice to generate CD45.1 cIAP1$^{-/-}$ OT-I mice. Mice were maintained under specific pathogen-free conditions in sterile microisolator cages. Animal studies were approved by the University of Toronto animal care committee in accordance with the regulations of the Canadian Council on Animal Care.

*CD8 T Cell Isolation and Proliferation Studies*—WT, TRAF1$^{-/-}$, or cIAP1$^{-/-}$ CD8 T cells were purified from spleens of naive mice using a negative selection mouse CD8 T cell enrichment kit (StemCell Technologies, Vancouver, Canada). In certain experiments, T cells were stained with 3 μM CFSE for 10 min at 37 °C. T cells ($1 \times 10^6$ cells/ml) were stimulated with immobilized monoclonal anti-murine CD3 (145-2C11; 1 μg/ml) in the presence or absence of plate-bound anti-4-1BB (3H3; 10 μg/ml; the 3H3 hybridoma was kindly provided by Robert Mittler, Emory University) or anti-CD28 (37.51; 10 μg/ml; kindly provided by Jim Allison, Memorial Sloan Kettering). Cell proliferation was determined by the progressive loss of CFSE signaling with each division. For small interfering RNA (siRNA) knockdown experiments, CD8 T cells were isolated and stained with CFSE as described above and $1 \times 10^6$ cells were transfected with 1 μM siRNA targeting NIK. Cells were rested for 12 h and then stimulated as described.

*Flow Cytometry*—CD8 T cells were surface stained with anti-CD8α (eBioscience, San Diego, CA) and in some cases a live/dead stain (Invitrogen). For intracellular cytokine staining, stimulated CD8 T cells were treated for 6 h with Golgi Stop (BD Biosciences, San Jose, CA) at 37 °C. Cells were surface stained, fixed, and intracellularly stained for IFN-γ, and TNF-α (BD Biosciences). Fluorescent minus one controls were used as negative controls. Samples were analyzed using a FACSCalibur (BD Biosciences) and FlowJo (Tree Star, Ashland, OR) software.

*Signaling Studies*—WT, TRAF1$^{-/-}$, or cIAP1$^{-/-}$ OT-I CD8 T cells were stimulated *in vitro* with 0.1 μg/ml SIINFEKL. Cells were subjected to lympholyte isolation (Cedarlane Laboratories, Hornby, Ontario, Canada) on day 3 of the culture, rested, and then stimulated with either 10 μg/ml agonist anti-4-1BB antibody, 3H3, or isotype control, rat IgG. For small interfering RNA (siRNA) knockdown experiments, cells were cultured for an additional 72 h in 20 ng/ml IL-15 (R&D Systems, Minneapolis, MN) after lympholyte isolation. A total of $3 \times 10^6$ OT-I cells were then transfected with 1 μM siRNA targeting cIAP2 or a control scrambled duplex RNA (IDT Technologies, Coralville, IA). Transfections were performed using the Amaxa mouse T cell Nucleofector kit (Lonza, Cologne, Germany). Cells were rested for 20 h and then stimulated with 10 μg/ml of either anti-4-1BB, 3H3, or isotype control, rat IgG.

*Western Blots*—Cells were lysed in lysis buffer (0.1% SDS, 1% TX-100, 150 mM NaCl, 20 mM Tris pH 7.4) with complete protease inhibitor mix (Roche, Basel, Switzerland). Lysates were quantified using a bicinchoninic acid protein assay kit (Thermo Fisher Scientific, Waltham, MA). Sample loading was normal-
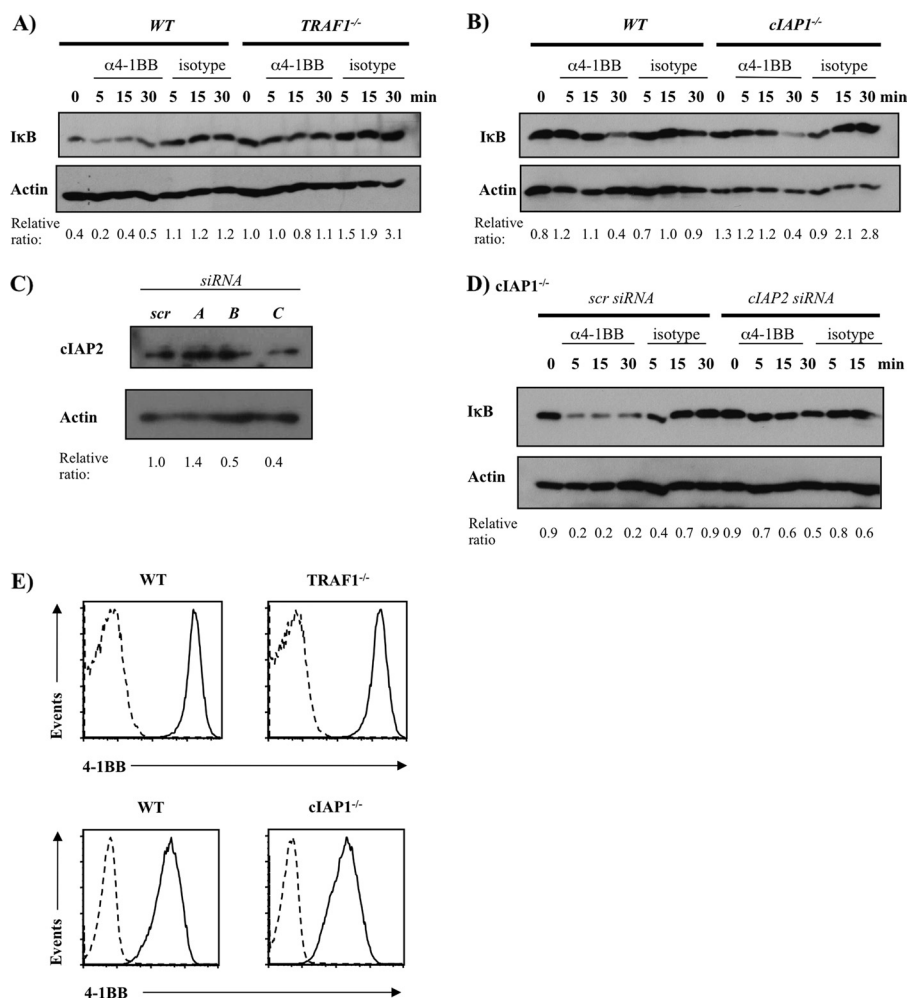
FIGURE 1. **4-1BB induced activation of the canonical NF-κB pathway requires TRAF1 and either cIAP1 or cIAP2.** WT, TRAF1$^{-/-}$, or cIAP1$^{-/-}$ OT-I splenocytes were stimulated with SIINFEKL peptide for 48 h and rested for 24 h. *A* and *B*, cells were stimulated in the presence of agonistic anti-4-1BB antibody or a rat isotype control for the indicated time and actin and IκB levels were analyzed in WT or TRAF1$^{-/-}$ T cells (*A*) or WT and cIAP$^{-/-}$ T cells (*B*) at the times indicated. *C*, cIAP1$^{-/-}$ OT-I T cells were further transfected with one of three variants of siRNA targeting cIAP2 or with scrambled RNA, and 20 h later cIAP2 protein levels were assessed by Western blot. *D*, C variant siRNA was used to knockdown cIAP2 expression and 20 h later IκB expression was assessed. *E*, activated OT-I CD8 T cells were isolated and 4-1BB expression determined. Data in *A–E* are representative of at least three independent experiments.

ized on the basis of protein content and samples were subjected to SDS-PAGE and then transferred to polyvinylidene difluoride membranes. Membranes were probed with Abs specific for IκBα, p100/p52, TRAF2, TRAF3, GAPDH (Cell Signaling Technology, Beverly, MA), or β-actin (Sigma-Aldrich) and subsequently incubated with HRP-conjugated anti-rabbit (Sigma-Aldrich). Signals were revealed by chemiluminescence (GE Healthcare, Baie D'Urfe, Quebec, Canada) and visualized by autoradiography. Where indicated, quantitation was performed using Quanitity One software (Bio-Rad).

## RESULTS

*TRAF1 and cIAPs Positively Regulate the Canonical NF-κB Pathway Downstream of 4-1BB*—To examine the role of TRAF1 in the activation of the canonical NF-κB pathway downstream of 4-1BB, we took advantage of TCR transgenic OT-I mice that had been crossed to TRAF1$^{-/-}$ mice (20). To study 4-1BB signaling on the T cells, we first activated the T cells with their peptide antigen to induce 4-1BB expression

then, following a period of rest, we engaged the T cells with anti-4-1BB antibody or isotype control. Stimulation of the WT antigen-activated T cells with anti-4-1BB led to activation of the classical NF-κB pathway as evidenced by IκB degradation (Fig. 1*A*). In contrast, the antigen activated TRAF1$^{-/-}$ T cells showed very little, if any, degradation of IκB in response to 4-1BB engagement (Fig. 1*A*). Thus TRAF1 is essential for 4-1BB induced activation of the canonical NF-κB pathway in primary activated T lymphocytes.

Recent data suggest that cIAP1 and cIAP2 are responsible for activation of the canonical NF-κB pathway downstream of TNFR family members, with the TRAFs serving as adaptors for their recruitment to TNFR signaling complexes (41). We therefore sought to determine if cIAP1 and cIAP2 have a role in 4-1BB induced NF-κB activation. We generated cIAP1$^{-/-}$ OT-I mice as a source of cIAP1$^{-/-}$ CD8 T cells. When cIAP1$^{-/-}$ OT-I CD8 T cells were stimulated with anti-4-1BB, IκBα degradation was observed with similar kinetics to that observed in WT OT-I CD8 T cells (Fig. 1*B*), suggesting that

cIAP1 is dispensable for 4-1BB induced NF-κB activation. However, it has been reported that cIAP1 and 2 play a redundant role in classical NF-κB activation downstream of TNFR1 (42). Thus to test the possibility that cIAP1 and 2 were also redundant downstream of 4-1BB signaling, we knocked down cIAP2 in the cIAP1$^{-/-}$ CD8 T cells. We first tested three different cIAP2 targeting siRNAs for their effects on cIAP2 expression in cIAP1$^{-/-}$ CD8 T cells. We found that cIAP2 siRNA C resulted in a substantial decrease in cIAP2 expression in cells (Fig. 1C) and used it for our subsequent cIAP2 knock down experiments. Indeed, when cIAP2 was knocked down in cIAP1$^{-/-}$ OT-I CD8 T cells, 4-1BB was no longer able to induce NF-κB activation, as observed by the lack of IκB degradation (Fig. 1D). Thus, cIAP1 and 2 play a redundant role in activation of the classical pathway downstream of 4-1BB in T cells.

To ensure that the effects were not due to differing expression of 4-1BB, we examined 4-1BB cell surface expression by flow cytometry and observed that WT, TRAF1$^{-/-}$, and cIAP1$^{-/-}$ OT-I CD8 T cells up-regulated 4-1BB to similar levels following antigen stimulation (Fig. 1E). Thus, TRAF1 and both cIAP1 and -2 have important roles in inducing classical NF-κB activation downstream of 4-1BB.

*TRAF1$^{-/-}$ CD8 T Cells Display Costimulation-independent Hyperproliferation and Effector Function*—The positive role for TRAF1 in 4-1BB costimulation induced classical NF-κB activation was surprising given that it had previously been reported that T cells lacking TRAF1 show increased proliferation to anti-CD3 stimulation alone (19). By labeling cells with CFSE and following their proliferation through CFSE dilution, we confirmed that TRAF1$^{-/-}$ CD8 T cells hyperproliferate to anti-CD3 alone and observed that the level of cell division was similar to that observed in WT CD8 T cells stimulated with anti-CD3 in the presence of costimulation (Fig. 2A). Costimulation through 4-1BB or anti-CD28 engagement did not lead to further enhancement of proliferation in the TRAF1$^{-/-}$ CD8 T cells (Fig. 2A). In addition, we observed an increase in the frequency of IFNγ- (Fig. 2B) and TNFα- (Fig. 2C) producing cells in the TRAF1$^{-/-}$ CD8 T cell cultures compared with WT CD8 T cells and this was also not further enhanced with costimulation. Thus, TRAF1$^{-/-}$ CD8 T cells display costimulation independent hyperproliferation and enhanced production of IFNγ and TNFα compared with WT CD8 T cells.

*4-1BB Activates the Alternative NF-κB Pathway in T Cells*—It was surprising that TRAF1$^{-/-}$ CD8 T cells do not show a defect in proliferation and cytokine production to 4-1BB costimulation as they have a defect in 4-1BB induced activation of the classical NF-κB pathway. Thus, we sought to investigate other pathways downstream of 4-1BB that could affect proliferation and cytokine production. Recently it has been reported that NIK, an activator of the alternative NF-κB pathway, is absolutely required for costimulation through the TNF receptor family member OX40 in graft-*versus*-host disease (43). A previous report showed that overexpresson of 4-1BB in 293 cells leads to the appearance of p52 (31). However, the activation of the alternative NF-κB pathway by 4-1BB has not been definitively examined in its physiological context. To test this, we isolated naïve CD8 T cells and stimulated them with either
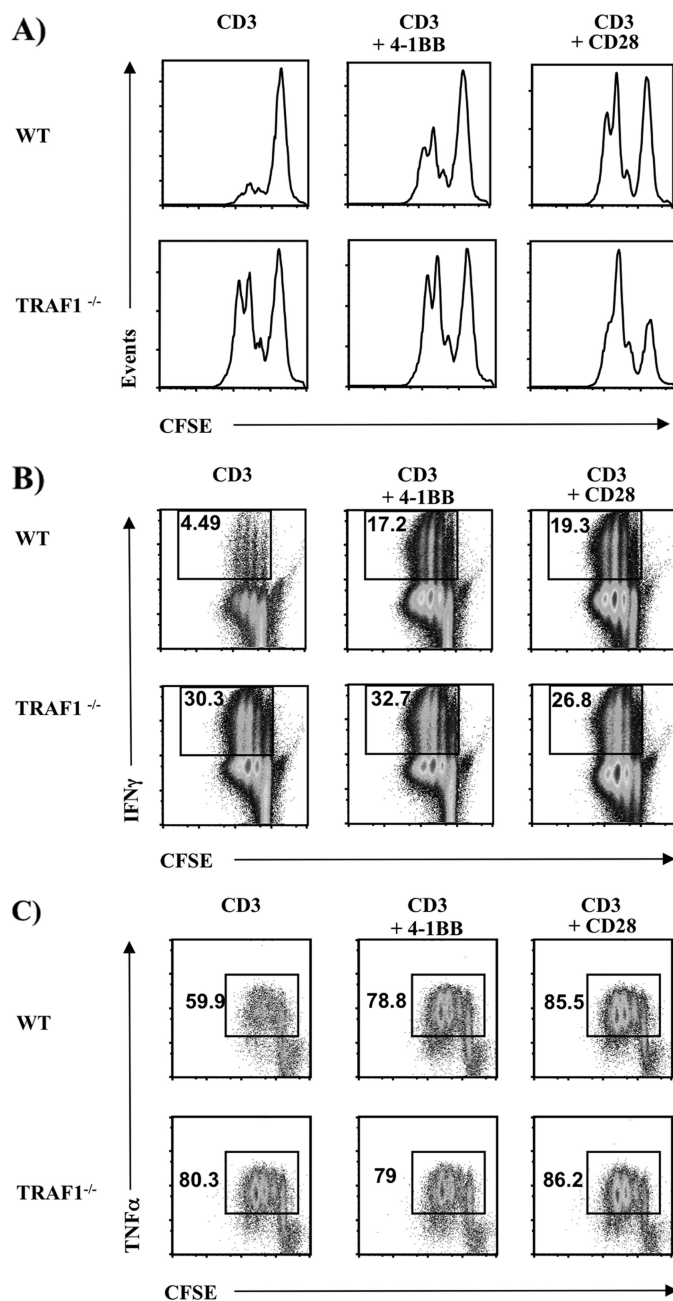


FIGURE 2. **Activated TRAF1$^{-/-}$ CD8 T cells display costimulation independent hyperproliferation and cytokine production.** Purified primary naïve CD8 T cells ($10^6$/ml) from WT and TRAF1$^{-/-}$ mice were CFSE labeled and stimulated for 2 days with the indicated agonistic antibodies. *A,* proliferation was measured by CFSE dilution, (*B*) IFNγ production and (*C*) TNFα production were measured by intracellular staining. The numbers in the *upper-boxed quadrant* indicate the percent of cells staining for the cytokine. Data are representative of at least three experiments.

anti-CD3 alone or anti-CD3 plus anti-4-1BB. We observed that anti-CD3 stimulation of the primary T cells led to up-regulation of p100 expression and that the addition of 4-1BB stimulation led to p100 processing to its active transcriptional regulatory fragment, p52 (Fig. 3A).

To further investigate the kinetics as well as mechanism of 4-1BB induced activation of the noncanonical NF-κB pathway, we used SIINFEKL activated OT-I CD8 T cells (as described in Fig. 1) and stimulated with either isotype control or with the
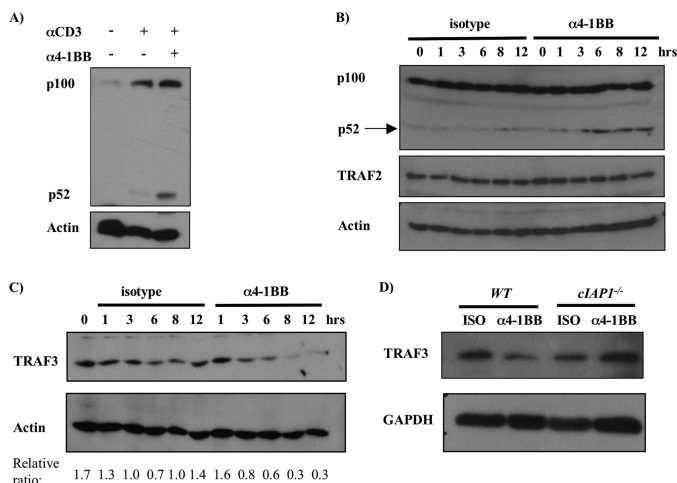
**FIGURE 3. 4-1BB signaling leads to cIAP1-dependent TRAF3 degradation and activation of the non-cononical NF-κB pathway.** *A*, purified primary CD8 T cells (10⁶/ml) from WT C57BL/6 mice were stimulated for 18 h with immobilized anti-CD3 (1 μg/ml) alone or with anti-CD3 plus immobilized anti- 4-1BB (10 μg/ml) or with anti-CD3 plus anti-CD28 (10 μg/ml) and lysates examined for p100 and p52 as well as actin. *B*, OT-I T cells were activated as in Fig. 1 and stimulated with agonistic anti-4-1BB antibody for the indicated time. Activation of alternative NF-κB was determined by Western blotting for p100/p52 as well as for levels of the upstream NF-κB regulators, TRAF2 (*B*) and TRAF3 (*C*). *D*, activated WT and cIAP1⁻/⁻ OT-I T cells were stimulated for 3 h and TRAF3 expression analyzed. Data are representative of at least three independent experiments.
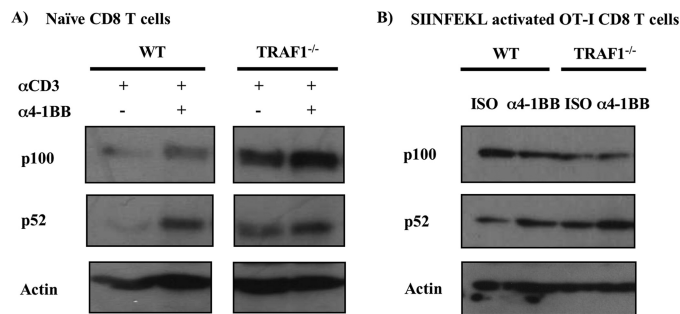


**FIGURE 4. Costimulation- independent activation of the alternative NF-κB pathway in CD8 T cells is restrained by TRAF1.** Activation of the alternative NF-κB pathway was determined by Western blotting for p100/p52. *A*, purified naïve CD8 T cells from WT and TRAF1⁻/⁻ mice were stimulated for 2 days with anti-CD3 and the indicated costimulatory antibodies. *B*, antigen-activated WT or TRAF1⁻/⁻ OT-I T cells, prepared as in Fig. 1, were further stimulated with agonistic anti-4-1BB antibody for 6 h and lysates analyzed for p100 processing to p52. Data are representative of three experiments.

agonistic anti-4-1BB antibody. By 6 h, activation of the noncanonical NF-κB pathway could be observed by the appearance of the p52 fragment in the 4-1BB stimulated samples (Fig. 3*B*). As well, although TRAF2 protein levels remained constant (Fig. 3*B*), TRAF3 loss could be observed 3 h after 4-1BB stimulation (Fig. 3*C*).

Of note, there is a delayed and very low level of p100 processing and TRAF3 degradation in the control rat Ig-treated samples. This may be due to the expression the TNFR family member, GITR and its ligand, GITRL, on activated T cells (44) resulting in a low level of GITR engagement. Hauer *et al.* (31) have shown that GITR can induce p100 processing which may explain this very low level activation of the noncanonical NF-κB pathway in the isotype control-treated samples at later time points.

It was previously shown that TRAF3 degradation downstream of CD40 is mediated by cIAP1 and cIAP2 (33, 34). Using cIAP1-deficient CD8 T cells, we show that 4-1BB is not able to induce TRAF3 degradation, indicating that 4-1BB induced TRAF3 degradation is dependent on cIAP1 (Fig. 3*D*). Thus, 4-1BB stimulation induces cIAP1-dependent degradation of TRAF3 and activation of the alternative NF-κB pathway.

*TRAF1 Regulates Activation of the Alternative NF-κB Pathway in Antigen-activated CD8 T Cells*—Given that TRAF1⁻/⁻ CD8 T cells display costimulation-independent proliferation and effector function despite a defect in the classical NF-κB pathway, we sought to determine the status of the alternative NF-κB pathway in TRAF1⁻/⁻ CD8 T cells. Naive CD8 T cells were isolated from WT and TRAF1⁻/⁻ mice and stimulated with either anti-CD3 alone or with anti-CD3 plus anti-4-1BB. Whereas WT CD8 T cells required anti-CD3 plus 4-1BB costimulation to induce the active p52 fragment, TRAF1⁻/⁻

CD8 T cells stimulated with anti-CD3 alone resulted in the appearance of the processed p52 protein (Fig. 4*A*). Moreover, in the TCR transgenic OT-I model, we observed that activated TRAF1⁻/⁻ CD8 T cells had constitutively higher levels of active p52 compared with WT CD8 T cells which require 4-1BB costimulation to observe this increase in p100 processing (Fig. 4*B*). Thus, in the absence of costimulation, TRAF1 has a role in restraining activation of the alternative NF-κB pathway in anti-CD3 (T cell receptor)-activated CD8 T cells.

*Noncanonical NF-κB Activation Is Required for the Hyperproliferation of TRAF1⁻/⁻ T Cells as Well as Their Increased Effector Function*—To determine whether the costimulation independent activation of the alternative NF-κB pathway in TRAF1⁻/⁻ CD8 T cells was responsible for their increased proliferation and cytokine production, we transfected primary WT and TRAF1⁻/⁻ CD8 T cells with scrambled or NIK-specific siRNA. Although NIK expression is difficult to detect in cells (45), we were able to confirm knockdown of NIK by looking at its function as a readout, *i.e.* the amount of p52 present (Fig. 5*A*). We observed that NIK knockdown in WT CD8 T cells resulted in reduced proliferation and cytokine production in cells stimulated with anti-CD3 alone but had no effect when exogenous costimulation (either anti-4-1BB or anti-CD28) was present (Fig. 5*B*). These results demonstrate that the alternative NF-κB pathway is responsible for enhancing proliferation as well as cytokine production in T cells stimulated in the absence of costimulation and suggests that when the alternative NF-κB pathway is compromised, costimulation through either 4-1BB or CD28 can provide additional signals to compensate. In contrast, knockdown of NIK in TRAF1⁻/⁻ CD8 T cells reduced the proliferation and cytokine production in response to anti-CD3 alone, as well as when 4-1BB costimulation was present (Fig. 5*C*). However, similar to WT CD8 T cells, TRAF1⁻/⁻ CD8 T cells costimulated with anti-CD28 were not affected by NIK knockdown, consistent with TRAF1 having no known function downstream of CD28. Thus, in contrast to WT CD8 T cells, 4-1BB costimulation in TRAF1⁻/⁻ CD8 T cells is not able to compensate for loss of the alternative NF-κB pathway and a defect in proliferation and cytokine production is observed.
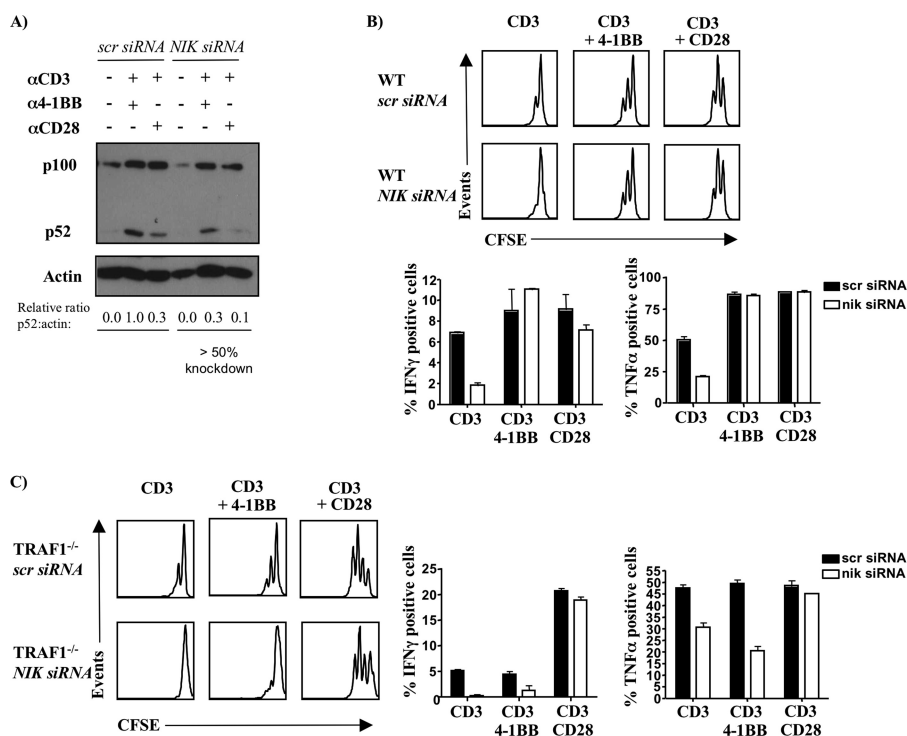
FIGURE 5. **Role of NIK in the proliferation and function of activated WT and TRAF1$^{-/-}$ CD8 T cells.** Purified naïve CD8 T cells from WT or TRAF1$^{-/-}$ mice were CFSE labeled and transfected with siRNA targeting NIK or a scrambled control. Cells were rested for 20 h and then stimulated for 2 days with the indicated agonistic antibodies. *A*, analysis of p100 processing in stimulated cells with or without NIK knockdown was analyzed by Western blot as a surrogate for measuring NIK levels. *B*, WT CD8 T cells and (*C*) TRAF1$^{-/-}$ CD8 T cells were transfected with scrambled siRNA or siRNA targeting NIK, proliferation of T cells was measured by CFSE dilution and IFNγ and TNFα production was measured by intracellular staining. The frequency of IFNγ and TNFα producing cells is shown as an average of three replicate wells. Data are representative of at least two experiments.

*cIAP1$^{-/-}$ CD8 T Cells Show Increased Effector Function but Still Respond to Costimulation*—CD8 T cells lacking cIAP1 can still activate the canonical NF-κB pathway downstream of 4-1BB (Fig. 1*D*) and cIAP1$^{-/-}$ MEFs have been reported to show constitutive p100 processing (37). Indeed, when we examined cIAP1$^{-/-}$ CD8 T cells, we observed that, similar to TRAF1$^{-/-}$ CD8 T cells, they displayed constitutive p100 processing with anti-CD3 stimulation alone (Fig. 6*A*). These results predict that cIAP1$^{-/-}$ CD8 T cells should be hyper-responsive to anti-CD3 stimulation but unlike TRAF1$^{-/-}$ CD8 T cells, should still be able to respond to 4-1BB costimulation. Indeed we observed that cIAP1$^{-/-}$ CD8 T cells hyper-proliferate in response to anti-CD3 stimulation compared with WT CD8 T cells (Fig. 6*B*). To examine the role of the alternative NF-κB pathway, NIK was knocked down in cIAP1$^{-/-}$ CD8 T cells prior to stimulation. Interestingly, knocking down NIK expression only dampened the cIAP1$^{-/-}$ CD8 T cell response to anti-CD3 stimulation but not to 4-1BB costimulation (Fig. 6*C*), similar to our findings with WT CD8 T cells (Fig. 5*B*). These data confirm at the functional level the non-redundant role of cIAP1 in restraining the alternative NF-κB pathway in activated T cells, but the redundant role for cIAP1 in 4-1BB-dependent costimulation, which in turn is able to compensate for loss of the alternative NF-κB pathway.

## DISCUSSION

The tight regulation of lymphocyte proliferation is essential to ensure appropriate expansion of lymphocytes during an immune response while limiting aberrant proliferation that could lead to inflammation and cancer. The requirement for T cells to receive both antigen specific signals through the TCR as well as costimulatory signals normally provided by CD28, provides a key control point for initiation of T cell proliferation (46). Members of the TNFR family provide a secondary control point in this process, by providing critical signals to prolong the survival of activated T cells (3, 8). In this report we show a key role for TRAF1 in CD8 T cells at these two critical check points: 1) We show that TRAF1, as well as cIAP1, play non-redundant roles in restraining costimulation independent activation of the non-canonical NF-κB pathway, as well as proliferation and cytokine production by antigen receptor (anti-CD3) activated CD8 T cells. 2) We also show that TRAF1, as well as cIAP1 or 2, are required for maximal 4-1BB induced canonical NF-κB activation, a pathway previously demonstrated as essential to 4-1BB-induced survival signaling in CD8 T cells (38). Taken together, these findings explain the opposing roles for TRAF1 in both prolonging T lymphocyte survival as well as restraining T cell proliferation until an appropriate costimulatory signal is received (18 –20).

Previous studies have shown a role for TRAF1 in 4-1BB-induced survival of CD8 T cells but have not addressed its role, or indeed that of cIAPs, specifically in 4-1BB-dependent NF-κB activation (17). Here we show that TRAF1, as well as cIAP1 or 2, are required for 4-1BB induced canonical NF-κB activation. It is unlikely that the defect in canonical NF-κB signaling in TRAF1$^{-/-}$ T cells is due to a generalized defect in TRAF1$^{-/-}$ T cells, as we previously showed that TRAF1 is dispensable for
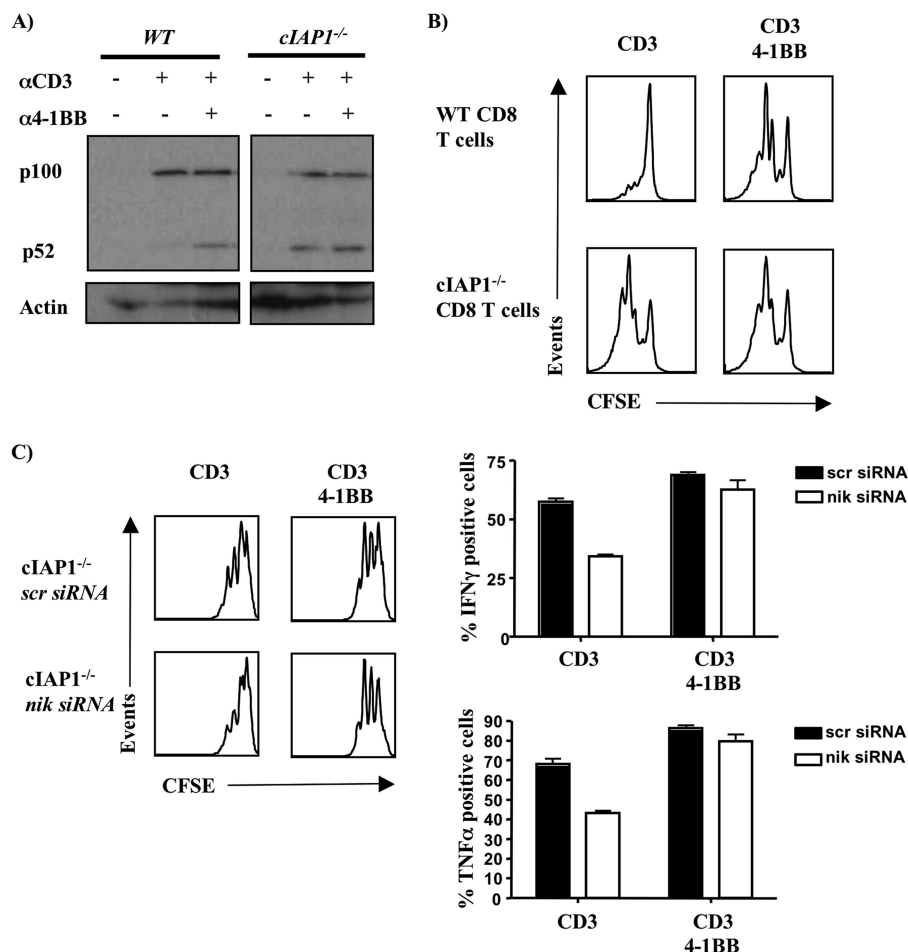
FIGURE 6. **cIAP1$^{-/-}$ CD8 T cells show increased effector function but still respond to costimulation.** *A*, purified primary CD8 T cells ($10^6$/ml) from WT and cIAP1$^{-/-}$ mice were stimulated for 18 h with immobilized anti-CD3 (1 $\mu$g/ml) alone or with anti-CD3 plus immobilized anti-4-1BB (10 $\mu$g/ml) or with anti-CD3 plus anti-CD28 (10 $\mu$g/ml) and lysates examined for p100 and p52 as well as actin expression. *B*, purified primary CD8 T cells from WT or cIAP1$^{-/-}$ mice were labeled with CFSE and stimulated as in *A* and cell division monitored by CFSE dilution. *C*, cIAP1$^{-/-}$ CD8 T cells were transfected with scrambled siRNA or siRNA targeting NIK, proliferation of T cells was measured by CFSE dilution and IFN$\gamma$ and TNF$\alpha$ production was measured by intracellular staining. The frequency of IFN$\gamma$ and TNF$\alpha$-producing cells is shown as an average of three replicate wells. Data are representative of at least two experiments.

activation of the canonical NF-κB pathway downstream of another TNFR member, GITR (6). In the case of GITR, we found instead that TRAF2 and TRAF5 cooperated in canonical NF-κB signaling (6), perhaps explaining why TRAF1 was not needed. Thus TRAF1 has a receptor-specific role in canonical NF-κB signaling downstream of 4-1BB.

Recent studies have elucidated the role of a complex consisting of TRAF2, TRAF3, and cIAP1 and -2 in restraining the alternative NF-κB pathway through constitutive NIK degradation (33, 34, 36). A recent study using an overexpression system, reported that TRAF1 and TRAF2 can be pulled down with TAP tagged NIK, suggesting that TRAF1 like TRAF2 might be part of the E3 complex for NIK (47). Here we have unconvered a hitherto unrecognized role for TRAF1 in contributing to inhibition of the non-canonical NF-κB pathway in TCR-activated CD8 T cells. Moreover, we show that TRAF1 deficiency and cIAP1 deficiency have a similar effect in inducing constitutive non-canonical NF-κB activation and T cell hyperproliferation as previously observed in studies that used SMAC mimetics to induce cIAP1 degradation (48).

We show that the costimulation-independent proliferation and cytokine production in WT, TRAF1$^{-/-}$, and cIAP1$^{-/-}$ T

cells is abrogated by knockdown of NIK. A number of earlier studies using NIK$^{aly/aly}$ and NIK$^{-/-}$ mice had indicated a critical function for NIK in T cell function (43, 49–53). However, a recent study by Hofmann *et al.* (54), showed that CD4 T cells from NIK$^{-/-}$ mice are anergic due to a role for NIK in thymic DCs rather than a T cell intrinsic role, leading to the suggestion that defects observed in T cells from NIK$^{aly/aly}$ and NIK$^{-/-}$ mice are due to a T cell developmental defect. In our model, using siRNA to knock down NIK expression in mature CD8 T cells, we ensure that the effects we are studying are due to a T cell intrinsic role for NIK. We observed that knockdown of NIK in WT CD8 T cells only affected CD8 T cell proliferation and cytokine production when T cells were stimulated in the absence of costimulation (Fig. 5), despite NIK being activated downstream of 4-1BB costimulation (Fig. 2). With the caveat that we haven't completely eliminated NIK in these knockdown experiments, the results show that in the presence of costimulation T cell intrinsic NIK may not be essential for CD8 T cell function, consistent with the results of Hofmann *et al.* (54). However, although some of the earlier studies (43, 49–53) may have reported defects that were due to a role for NIK in T cell development, our results (Fig. 5) clearly show that T cell intrin-

sic NIK can still affect proliferation and cytokine production in T cells activated in the absence of strong costimulation.

In contrast to WT and cIAP1$^{-/-}$ T cells, TRAF1$^{-/-}$ T cells are not able to respond normally to 4-1BB costimulation in the absence of non-canonical NF-κB. Stabilization of NIK is also able to induce classical NF-κB signaling by activating the upstream kinase IKKβ (55, 56) and through regulating p100, which in turn regulates the nuclear localization of RelA in addition to RelB (57). Thus, at least some of the effects of the alternative NF-κB pathway on T cell function may be due to it regulating the classical NF-κB pathway. Indeed, this would explain why WT and cIAP1$^{-/-}$ CD8 T cells do not require the non-canonical NF-κB pathway when 4-1BB costimulation is present, but TRAF1$^{-/-}$ T cells do.

Whereas the present study shows that TRAF1 is critical to prevent NIK activation in activated T cells, Zheng *et al.* did not observe constitutive processing of p100 to p52 in TRAF1$^{-/-}$-resting B cells (47). As resting B cells, and in fact most non-lymphoid cells, lack detectable TRAF1 (58), the regulation of NIK in resting primary B cells and other cell types could not involve TRAF1 or these cells would show constitutive alternative NF-κB activation. Thus the initial activation of the T cells may up-regulate components of the pathway sufficiently to require additional constraints on the alternative NF-κB pathway. Indeed, we observed that p100 expression is up-regulated by TCR signaling and that once induced in the antigen-activated T cells, TRAF1$^{-/-}$ T cells showed increased p100 processing to p52, leading to their increased production of cytokines and hyperproliferation. On the other hand, we were unable to detect increased NIK protein or mRNA expression in TRAF1$^{-/-}$ primary CD8 T cells (data not shown).

Although not fully explained, our results point to a fundamental difference in the requirement for TRAF1 for negative regulation of NIK in antigen receptor-activated T cells compared with resting T or B cells and other TRAF1-negative cells. Consistent with this finding, the non-canonical NF-κB pathway has been shown to have distinct functions in B cells compared with T cells: Whereas TRAF2- and TRAF3-deficient B cells accumulate and have increased survival, the TRAF2- and TRAF3-deficient T cells do not despite both showing constitutive non-canonical NF-κB activation (59, 60). In fact the TRAF3-deficient T cells actually show decreased proliferation in response to anti-CD3 stimulation, attributed to TRAF3 having a role in T cell receptor signaling (60).

In contrast to our study, others have observed a positive role for TRAF1 in activating the non-canonical NF-κB pathway in B cell-derived Hodgkin's lymphoma cell lines L1236 (61), L428, and KM-H2 (62). As Hodgkin's lymphomas constitutively express the EBV protein LMP1 and some express CD30, both of which can show ligand-independent signaling (62–64) it is possible that the differential role of TRAF1 downstream of these TNFRs explains why TRAF1 had apparently differing roles in lymphoma compared with primary T cells studied here. Thus the role for TRAF1 in restraining the alternative NF-κB pathway may be cell type and context specific.

Recently it was demonstrated that TRAF1 and TRAF2 preferentially form a TRAF1(TRAF2)$_2$ heterotrimer, which interacts with a single cIAP molecule with a much higher affinity than a TRAF2 homotrimer (47). This suggests that TRAF1, itself an NF-κB induced protein, may have an important role in the feedback enhancement of TRAF2 dependent signaling downstream of TNFR family members. Our data lend further support for this model in showing that TRAF1 is important in classical NF-κB signaling downstream of 4-1BB. Similar findings have been made by Arron *et al.* (21) and Xie *et al.* (22) for CD40 signaling, wherein, TRAF1 was found to sustain TRAF2 dependent signaling through CD40. Our data also extend to the 4-1BB signaling pathway the requirement for either cIAP1 or -2 for activation of the classical NF-κB pathway. The redundancy of cIAP1 and -2 induction of the classical NF-κB pathway downstream of 4-1BB signaling fits with the structural data that the TRAF1/2 complex asymetrically recruits one cIAP BIR domain (47) and is similar to the redundant role for cIAP1 and 2 in the classical pathway downstream of TNFR1 (42). On the other hand, deficiency of cIAP1 alone is sufficient to impair TRAF3 degradation downstream of 4-1BB, implying a non-redundant role of cIAP1 in induction of the non-classical NF-κB pathway downstream of 4-1BB (Fig. 2*B*). Moreover, our data also confirm previous observations that knockdown of only cIAP1 in T cells is sufficient to allow hyperproliferation in response to anti-CD3, again indicating a non-redundant role of cIAP1 in this process (48).

In conclusion, there have been conflicting data as to whether TRAF1 is a positive or negative regulator of TNFR signaling. Our study clarifies this issue by showing that TRAF1 can restrain the alternative NF-κB pathway in antigen-activated T cells, whereas it is essential for maximal 4-1BB dependent activation of the classical NF-κB pathway. Thus TRAF1 plays a key role at two checkpoints in T cell activation, first in restraining costimulation independent T cell activation and then in promoting TNFR-dependent survival signaling in activated T cells.

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