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ERK-Dependent Bim Modulation Downstream of the 4-1BB-TRAF1 Signaling Axis Is a Critical Mediator of CD8 T Cell Survival In Vivo¹ ✓

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ERK-Dependent Bim Modulation Downstream of the 4-1BB-TRAF1 Signaling Axis Is a Critical Mediator of CD8 T Cell Survival In Vivo¹

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During an acute immune response, CD8 T cells undergo rapid expansion followed by a contraction phase during which the majority of activated T cells die, leaving a few survivors to persist as memory cells. The regulation of T cell survival is critical at each stage of this response. 4-1BB, a TNFR family member, has been implicated in prolonging the survival of activated and memory CD8 T cells; however, the precise mechanisms by which 4-1BB sustains T cell survival are incompletely understood. Upon aggregation on T cells, 4-1BB associates with two TNFR-associated factors (TRAF), TRAF1 and TRAF2. TRAF2 is essential for downstream signaling from 4-1BB; however, the role of TRAF1 in 4-1BB signaling has not been elucidated and there have been conflicting data as to whether TRAF1 provides a positive or a negative signal in T cells. In this study, we report that TRAF1 plays a critical role in survival signaling downstream of 4-1BB during CD8 T cell expansion in response to viral infection in vivo. Further analysis reveals that TRAF1-deficient cells are impaired in their ability to up-regulate the prosurvival Bcl-2 family member Bcl-x_L and show increased levels of the proapoptotic Bcl-2 family member Bim following 4-1BB signaling. TRAF1-deficient CD8 T cells fail to activate ERK in response to 4-1BB ligation and inhibition of ERK signaling downstream of 4-1BB in wild-type cells leads to increased Bim levels. Thus, TRAF1 has a prosurvival effect in CD8 T cells via the 4-1BB-mediated up-regulation of Bcl-x_L and ERK-dependent Bim down-modulation. *The Journal of Immunology*, 2008, 180: 8093–8101.

Activation of T cells requires engagement of the TCR by MHC/peptide, which confers specificity. However, complete activation of T cells depends upon additional signals that allow the cells to produce cytokines, proliferate, and survive. Although CD28 provides the primary costimulatory signal, several members of the TNFR family, such as 4-1BB, OX40, TNFR2, and CD27, provide signals to sustain T cell responses (1–3). The 4-1BB costimulatory pathway plays a key role in T cell survival and CD8 T cell memory (4–10).

In T cells, 4-1BB signaling is associated with the recruitment of TNFR-associated factors (TRAF)³ 1 and 2 (11). TRAF2 links 4-1BB to downstream JNK/stress-activated protein kinase (12), p38 MAPK (13), and NF-κB (14–16) pathways resulting in proliferation, cytokine production, and survival. Although an essential role for TRAF2 downstream of 4-1BB ligation has been demonstrated (11, 13), the role of TRAF1 in 4-1BB-dependent signaling has not been elucidated.

TRAF proteins share a conserved C-terminal TRAF domain, the coiled-coil TRAF-N region that permits TRAF oligomerization and a TRAF-C region responsible for association with TRAF-binding motifs in TNFR cytoplasmic tails (17). The N-terminal domains of most TRAF proteins contain a series of zinc-binding domains required for linkage to the stress-activated protein kinase cascade and a RING finger domain responsible for E3 ubiquitin ligase activity and linkage to the NF-κB signaling pathway (18–22). TRAF1 differs from the other TRAF family members in lacking the RING finger domain and containing only one Zn finger, thus resembling a dominant negative form of TRAF2 (20). Indeed, in overexpression systems, TRAF1 can limit signaling via TRAF2 (23, 24).

In contrast to results obtained from overexpression studies, the generation of TRAF1-deficient mice and cell lines demonstrated that TRAF1 is required for maximal TRAF2-dependent signaling through CD40 in dendritic cells and B cells (21, 25). In contrast, the hyper-responsiveness of TRAF1-deficient T cells to TNF or Ag-receptor signaling suggests that TRAF1 negatively regulates signaling through TNFR2 and the TCR (26, 27). Thus, the effects of TRAF1 may be receptor and/or cell specific. Several members of the TNFR family expressed on T cells can recruit TRAF1, including TNFR2, 4-1BB, glucocorticoid-induced TNF receptor, and CD30 (reviewed in Ref. 28). Recent data from our laboratory established that TRAF1 plays a key role in survival of activated and memory CD8 T cells (29); however, the specific TNFR family members involved in this TRAF1-dependent survival signaling were not determined. Given its preferential effects on CD8 T cell survival (4, 8, 10, 30), 4-1BB is a prime candidate for a TRAF1-binding receptor that maintains CD8 T cell survival.

In this study, we use TRAF1-deficient T cells to explore the role of TRAF1 in signaling via 4-1BB in vivo and in vitro using three distinct model systems. Although hyper-responsiveness of

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³ Abbreviations used in this paper: TRAF, TNFR-associated factor; WT, wild type; HAU, hemagglutinin unit.

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TRAF1^{-/-} T cells was observed in response to anti-CD3 stimulation alone, in the presence of 4-1BB signaling the dominant effect of TRAF1 is as a prosurvival molecule in CD8 T cells. Wild type (WT) T cells were found to increase Bcl-x_L expression and down-regulate the expression of the proapoptotic BH3-only family member Bim in response to 4-1BB costimulation. In contrast, TRAF1-deficient T cells show reduced up-regulation of Bcl-x_L and fail to down-regulate Bim, consistent with decreased T cell recovery compared with WT T cells. We further demonstrate that the mechanism by which TRAF1 maintains low levels of Bim in 4-1BB stimulated CD8 T cells is via 4-1BB-dependent ERK activation, leading to Bim down-modulation. Thus TRAF1 has a clear prosurvival role downstream of 4-1BB signaling in CD8 T cells, through up-regulation of Bcl-x_L and ERK-dependent down-modulation of Bim.

Materials and Methods

Mice

C57BL/6 mice were obtained from Charles River Laboratories. OT-I mice (31) were obtained from The Jackson Laboratory. TRAF1^{-/-} gene-targeted mice (26) backcrossed onto C57BL/6 background ($n = 8$), as well as OT-I TRAF1^{-/-} mice (29), have been described. 4-1BB^{-/-} mice, backcrossed onto C57BL/6 background ($n = 9$) (32) were provided by Byoung S. Kwon (Louisiana State University, New Orleans, LA) and were used directly or after crossing with OT-I TCR transgenic mice, for use in adoptive transfer experiments. All studies involving mice were in accordance with the regulations of the Canadian Council on animal care.

Lymphocyte isolation and stimulation

T cells were isolated from mouse spleens and lymph nodes by negative depletion using magnetic bead separation (Easy Sep; StemCell Technologies), purity was >95%. T cells (1×10^6 cells/ml) were stimulated with immobilized monoclonal anti-murine CD3 ($1 \mu\text{g/ml}$) in the presence or absence of plate-bound 4-1BBL extracellular domain ($10 \mu\text{g/ml}$) or anti-CD28 ($10 \mu\text{g/ml}$). The extra-cellular domain of 4-1BBL expressed in baculovirus construct was purified from insect cells as previously described (5, 11). To assay IL-2 production, supernatants were collected at 48 h and serial dilutions were cultured with an IL-2-dependent cell line, CTLL, as previously described (33). In other experiments, we used anti-CD3 ($1 \mu\text{g/ml}$) and anti-4-1BB (3H3; $10 \mu\text{g/ml}$) (30). Alloresponses used 10^5 MHC-I⁺ P815 mastocytoma (H-2^b) cells with or without transfected murine 4-1BBL and irradiated (9000 rads) before use as stimulators. The responders were 10^6 T cells purified from C57BL/6 WT or TRAF1^{-/-} (H-2^b) spleens. T cells were CFSE labeled where indicated. For ERK1/2 inhibition, T cells were incubated with $10 \mu\text{M}$ U0126 (Promega) for 30 min before stimulation with anti-4-1BB.

For *in vivo* analysis, 2×10^3 purified T cells, each from WT CD45.1 OT-I and TRAF1^{-/-} Thy1.1 OT-I mice, were mixed to a 1:1 ratio and injected *i.v.* into C57BL/6 Thy1.2, CD45.2 congenic mice. Twenty-four hours later, mice were infected *i.p.* with 64 hemagglutinin unit (HAU) of Flu-OVA (WSN-OVA), an influenza recombinant expressing the SIINFEKL epitope in its neuraminidase stalk (34) together with $100 \mu\text{g}$ of anti-4-1BB (3H3) (30) or $100 \mu\text{g}$ rat IgG (Sigma-Aldrich). CD45.1 and Thy1.1 T cell recovery was monitored as described in the figure legends. For analysis of the effect of TRAF1 on early division in response to 4-1BB signaling *in vivo*, 10^6 CFSE-labeled WT or TRAF1^{-/-} OT-I cells were injected into mice and immunized as above, and CFSE levels in the transferred T cells analyzed 3 days postimmunization.

Flow cytometry

The following anti-mouse mAbs were used: anti-CD8 α -allophycocyanin and anti-4-1BB-PE (ebioscience). For intracellular staining, cells were fixed in 2% formaldehyde for 10 min at 37°C, surface-stained, permeabilized with 90% cold methanol overnight at -20°C. Cells were stained with anti-mouse Bcl-x_L-FITC (Cell Signaling Technology) or $1 \mu\text{g}$ of rat anti-Bim (Alexis Biochemicals) or anti-mouse phospho-ERK1/2-PE (pT202/pY204, phosphoflow reagent; BD Biosciences) for 1 h at room temperature. Anti-Bim was detected with anti-rat-FITC (BD Biosciences; 30 min at room temperature). For CFSE staining, purified T cells were washed twice with PBS and resuspended in prewarmed PBS at a concentration of 5×10^7 cells/ml with $2.5 \mu\text{M}$ CFSE (Molecular Probes) and incubated at 37°C for 15 min. Cells were then washed once in media with 10% FCS to bind excess CFSE.

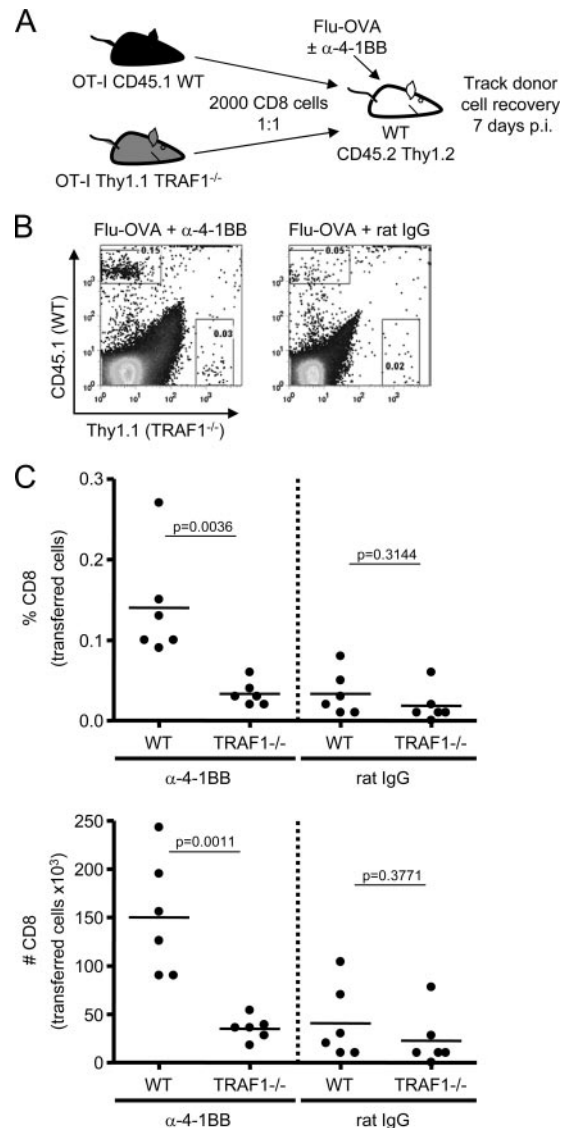


FIGURE 1. Role of TRAF1 in 4-1BB-dependent T cell expansion *in vivo*. *A*, Naive purified CD8 T cells from OT-I CD45.1 WT and OT-I Thy1.1 TRAF1^{-/-} mice (2000 each, 1:1 ratio) were transferred into naive CD45.2 Thy1.2 mice. Twenty-four hours later, mice were immunized *i.p.* with 64 HAU Flu-OVA together with $100 \mu\text{g}$ agonist anti-4-1BB Ab or $100 \mu\text{g}$ control rat IgG Ab. Seven days postinfection, spleens were isolated and recovery of transferred cells was determined. *B*, Representative FACS plots of live CD8 T cells isolated 7 days postinfection. *C*, Summary of one experiment with six mice per group, showing % live CD8 and total numbers of CD8 transferred cells recovered. Similar results were obtained in a second experiment with three to five mice per group.

Western blot analysis

Cell lysates of live activated OT-I CD8 T cells expressing surface 4-1BB, were prepared using SIINFEKL ($0.1 \mu\text{g/ml}$), as described in the figure legends, followed by lymphocyte purification, as directed by the manufacturer (Cedarlane Laboratories). After stimulation with anti-4-1BB, 10^6 cells were washed in cold PBS, lysed in protein loading buffer and subjected to SDS-PAGE, and then transferred to polyvinylidene difluoride membranes (Pall Life Sciences). Membranes were probed with Abs specific for Bcl-2 (Santa Cruz Biotechnology), Bim, and TRAF2 (Cell Signaling Technology), total and phospho-ERK1/2 (pT202/pY204; Cell Signaling Technology), or β -actin (Sigma-Aldrich) and incubated with the HRP-conjugated anti-rabbit Ig Ab (Sigma-Aldrich). Signals were revealed by chemiluminescence (Amersham Biosciences) and visualized by autoradiography. Densitometry was performed using the Quantity One software (Bio-Rad).

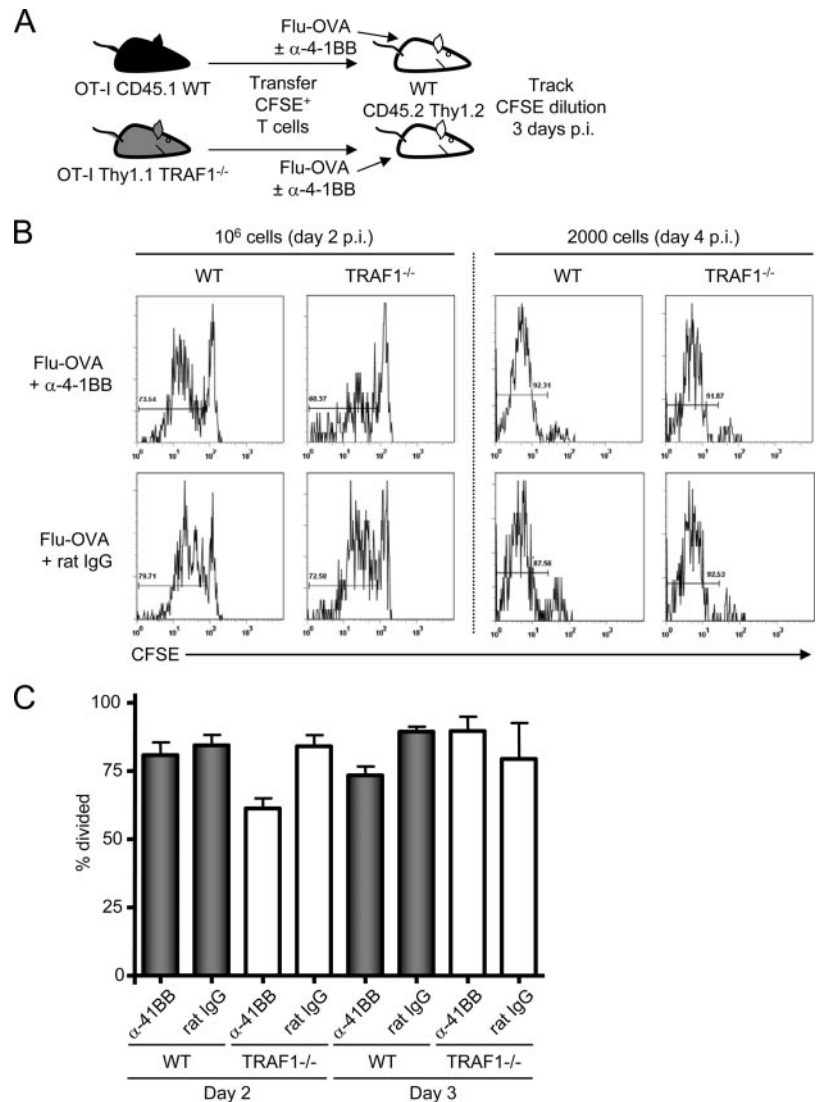


FIGURE 2. 4-1BB and TRAF1 do not influence the rate of division of CD8 T cells in response to influenza infection in vivo. *A*, Naive CD8 T cells were purified from OT-I WT and OT-I TRAF1^{-/-} mice, labeled with CFSE and either 10⁶ or 2000 cells were transferred into a naive host. Twenty-four hours later, mice were immunized i.p. with 64 HAU Flu-OVA plus 100 μg anti-4-1BB stimulatory Ab or control rat IgG Ab. *B*, At 2 and 3 days post infection for the 10⁶ transferred cells, or 4 days postinfection for 2000 transferred cells, spleens were isolated and CFSE fluorescence of the congenically marked transferred cells was determined. Flow cytometry plots shown represent the plot showing the median amount of division of three to five mice per group. *C*, Summary of results at 2 and 3 days postinfection for three to five mice per group after 10⁶ OT-I T cells were transferred. These data are representative of two similar experiments.

Results

TRAF1 is required for survival of T cells following expansion in the presence of anti-4-1BB in vivo

To explore the role of TRAF1 in a 4-1BB-mediated T cell response in vivo, we used an adoptive transfer model with CD8 OVA_{257–264}-specific TCR transgenic OT-I cells. A 1:1 mixture of purified OT-I CD45.1 WT T cells and OT-I Thy1.1 TRAF1^{-/-} T cells were transferred into CD45.2/Thy1.2 C57BL/6 naive mice and their expansion was monitored following immunization with an influenza virus carrying the SIINFEKL epitope in its neuraminidase stalk (Flu-OVA) (Fig. 1). As previously described, during infection with influenza virus, initial CD8 T cell expansion can be enhanced by simultaneous injection of anti-4-1BB Ab (35, 36). When mice were infected with Flu-OVA plus a control rat IgG Ab, the level of expansion of WT vs TRAF1^{-/-} OT-I T cells was minimal and not significantly different. Inclusion of anti-4-1BB during the immunization resulted in a greatly increased recovery of OT-I T cells, but failed to increase the recovery of the OT-I TRAF1^{-/-} T cells (Fig. 1). The increased recovery of TRAF1-sufficient T cells is unlikely due to trafficking or kinetic differences, as previous studies showed similar defects in CD8 T cell responses to influenza virus in TRAF1-deficient mice in lung, spleen, and lymph node at several time points throughout the primary and secondary response (29). Furthermore, in the current experiments, similar results were

obtained in spleen (Fig. 1) and lymph node (data not shown). 4-1BB^{-/-} OT-I T cells showed no response to anti-4-1BB in this model (data not shown). These results show that TRAF1 in the T cells is required for the maximal recovery of T cells in response to 4-1BB signaling in vivo.

The role of TRAF1 on T cell recovery during 4-1BB stimulation in vivo could be due to effects on T cell division, T cell survival, or both. To address this issue, WT and TRAF1^{-/-} OT-I T cells were labeled with CFSE and cell division was assessed by flow cytometry (Fig. 2*A*) at days 2 and 3 following immunization with Flu-OVA in the presence or absence of anti-4-1BB when 10⁶ cells were transferred or at day 4 with 2000 transferred cells (Fig. 2, *B* and *C*). There was a similar level of division of the T cells whether the mice were treated with control rat IgG or anti-4-1BB and whether the T cells expressed TRAF1 or not (Fig. 2, *B* and *C*). This experiment suggests that 4-1BB and TRAF1 cooperate in the T cells to allow maximal survival in vivo, independently of effects on cell division.

ERK-dependent Bim down-modulation in OT-I T cells following 4-1BB signaling

In activated T cells, the relative expression of the proapoptotic BH3-only family member Bim, compared with the anti-apoptotic

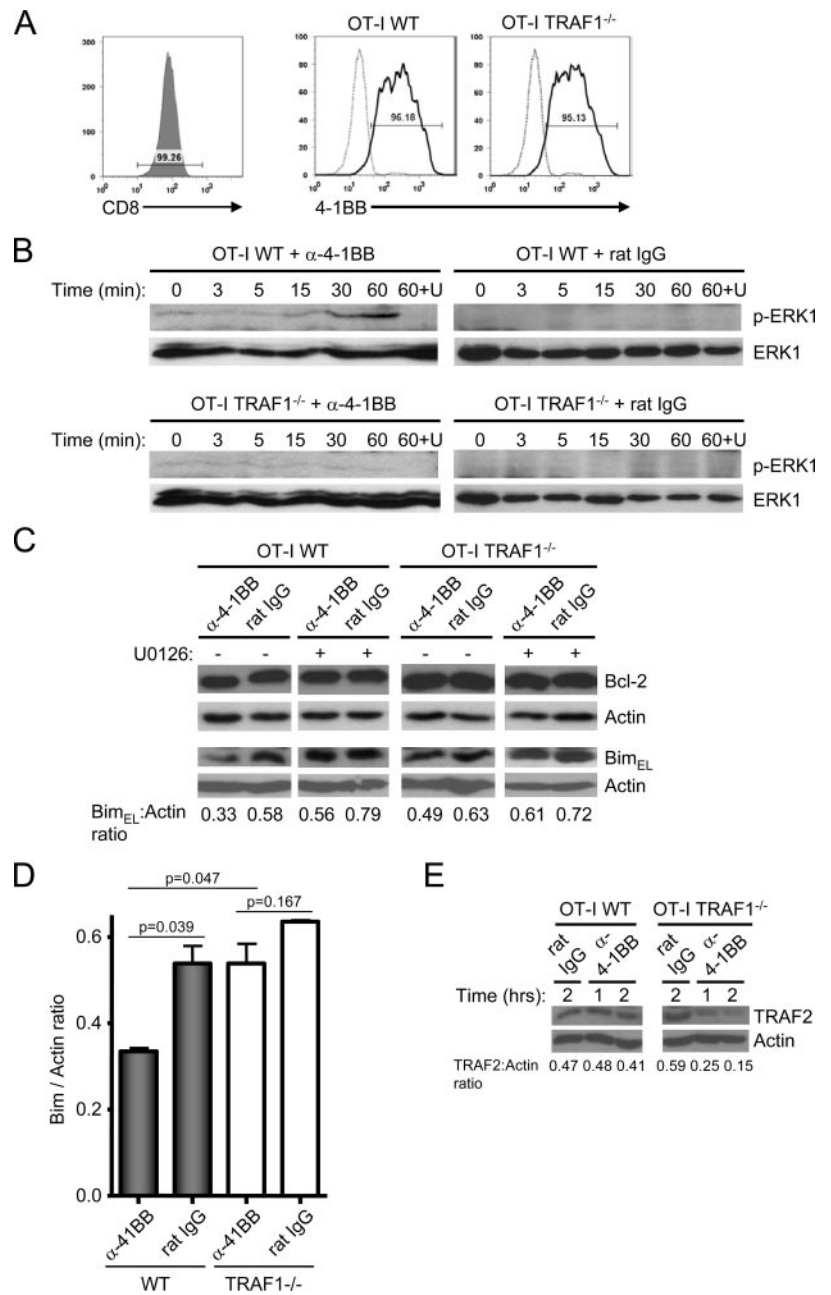


FIGURE 3. TRAF1-dependent ERK activation downstream of 4-1BB signaling results in lower levels of Bim in activated T cells. OT-I splenocytes (WT and TRAF1^{-/-}) were stimulated with 0.1 μ g/ml SIINFEKL peptide for 48 h. **A**, Live CD8 T cells were isolated from the cultures and 4-1BB expression was confirmed. Dotted lines represent isotype control staining. **B**, The purified Ag-activated OT-I T cells were then incubated with 10 μ g/ml anti-4-1BB (*left*) or control rat IgG (*right*) in the presence or absence of the ERK1/2 specific inhibitor U0126 (U, 25 μ M) for the times indicated. Phospho-ERK and total ERK levels were then determined by Western blot. **C**, Purified, 48-h Ag-activated OT-I T cells were incubated on plate-bound anti-4-1BB (10 μ g/ml, 4-1BB or control rat IgG) for 18 h and lysates monitored for Bim, Bcl-2, and actin. **D**, Summary of Bim:actin ratios from two experiments of **C**. **E**, TRAF2 levels were monitored in OT-I (activated as described above) cells by Western blot (representative of two experiments).

molecules Bcl-2 and Bcl-x_L, is a critical determinant of their survival (37, 38). Previous work from our laboratory reported the requirement for TRAF1 to maintain low levels of Bim in activated and memory T cells (29); however, neither the TNFR family members responsible for this signaling nor the mechanism by which TRAF1 regulates Bim were determined. Bim is regulated in lymphocytes by the FOXO-3A transcription factor. Following cytokine withdrawal, the PI3K pathway is inhibited, which leads to inactivation of PKB, dephosphorylation of FOXO-3A, and its entry into the nucleus where it induces Bim transcription (39). In addition, Bim is regulated in cells by ERK phosphorylation (40). Activated ERK can phosphorylate Bim at several sites and cause its proteasome-dependent degradation, independently of the effects of PI3K and FOXO-3A (40, 41).

To explore the role of ERK and TRAF1 in regulating Bim levels following 4-1BB signaling, WT or TRAF1^{-/-} OT-I splenocytes were stimulated with their peptide Ag for 18 h, after which excess peptide was washed away and the cells were rested for 24 h. At the end of this

stimulation, CD8 T cells from WT or TRAF1^{-/-} OT-I cultures were isolated and found to uniformly express 4-1BB (Fig. 3A). The Ag-activated OT-I T cells were then stimulated with anti-4-1BB and ERK activation was monitored by Western blot analysis for phospho-ERK1 (p-ERK1) (Fig. 3B). 4-1BB was found to increase the levels of p-ERK1 as early as 30-min poststimulation, an effect that was blocked by the ERK inhibitor U0126 (Fig. 3B). In contrast, TRAF1-deficient Ag-activated OT-I T cells failed to activate ERK in response to 4-1BB signaling (Fig. 3B, lower panel), despite equivalent expression of 4-1BB on the Ag-activated T cells (Fig. 3A, right).

We next analyzed the effect of 4-1BB stimulation and ERK inhibition on Bim levels in Ag-activated OT-I T cells (Fig. 3, C and D). In WT Ag-activated OT-I T cells, stimulation with plate bound anti-4-1BB led to reduced levels of Bim compared with control (rat IgG)-treated Ag-activated OT-I T cells. Furthermore, inhibition of ERK with U0126 during 4-1BB stimulation resulted in higher levels of Bim. In contrast, 48-h Ag-activated TRAF1-deficient OT-I T cells showed minimal down-regulation of Bim in

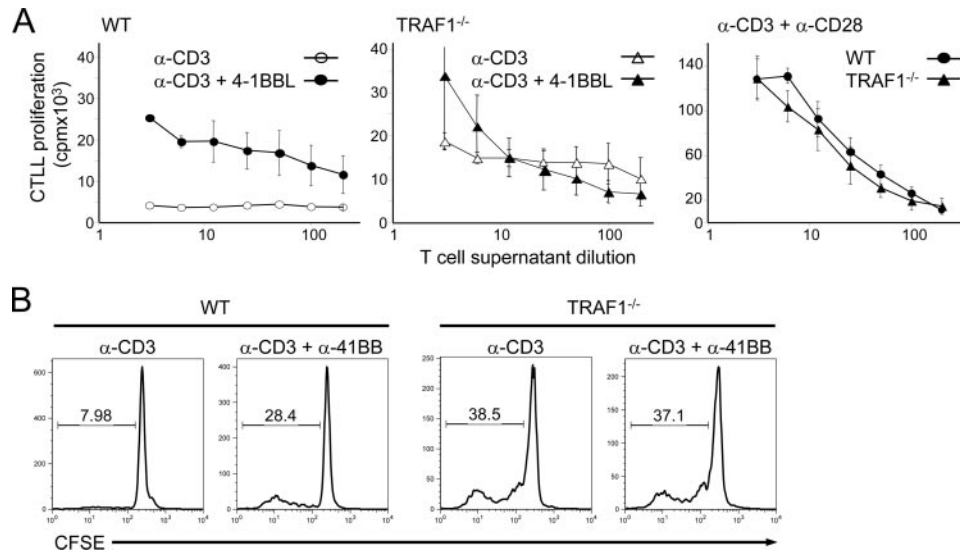


FIGURE 4. Enhanced proliferation to anti-CD3 but failure to respond to 4-1BB stimulation in TRAF1-deficient T cells. *A*, Purified primary T cells ($10^6/ml$) from WT or TRAF1^{-/-} mice were stimulated for 48 h with immobilized anti-CD3 (1 $\mu g/ml$) alone or with immobilized 4-1BBL (10 $\mu g/ml$) or anti-CD3 plus anti-CD28 (10 $\mu g/ml$) as a positive control. IL-2 production is plotted as averaged triplicates of [³H]thymidine incorporation by the IL-2-dependent cell line CTLL in response to serial dilutions of the supernatants from the stimulated T cell cultures. Note that 4-1BBL alone or anti-CD28 alone gave background response (not shown). *B*, Purified T cells from WT or TRAF1^{-/-} mice were stained with CFSE, and then incubated with immobilized anti-CD3 (1 $\mu g/ml$) alone or with immobilized anti-4-1BB (10 $\mu g/ml$) for 48 h. These data are representative of two similar experiments.

response to 4-1BB signaling. As an additional control, we also monitored Bcl-2 levels, as previous results have shown Bcl-2 levels do not change in the absence of TRAF1 (29) or during 4-1BB signaling (42). As expected, Bcl-2 levels were unchanged by 4-1BB stimulation or by ERK inhibition. Thus, 4-1BB signaling leads to TRAF1-dependent ERK activation, which contributes to the maintenance of lower levels of Bim in the activated T cells.

Previous studies have shown a role for TRAF1 in maintaining TRAF2 levels in cells downstream of CD40 signaling (21, 25). To determine whether TRAF1 influenced TRAF2 levels in the cells, we analyzed TRAF2 levels by Western blot after control Ab or 1 and 2 h after anti-4-1BB treatment of Ag-activated WT and TRAF1-deficient OT-I T cells. Indeed, levels of TRAF2 were

lower in the absence of TRAF1 (Fig. 3E). Thus, the effect of TRAF1 on Bim levels and T cell survival could be direct or could be indirect via effects on TRAF2.

Increased proliferation and IL-2 production in response to TCR signaling, but lack of 4-1BB-mediated responses, in TRAF1-deficient T cells

Previous studies have shown that TRAF1-deficient T cells show enhanced proliferation in response to signals through the TCR (26, 27). However, this hyperproliferation was not apparent in the OT-I model studied here. To more clearly segregate the effect of TRAF1 on TCR vs 4-1BB-mediated signals, we used a costimulation model in which purified T cells are stimulated with plate-bound

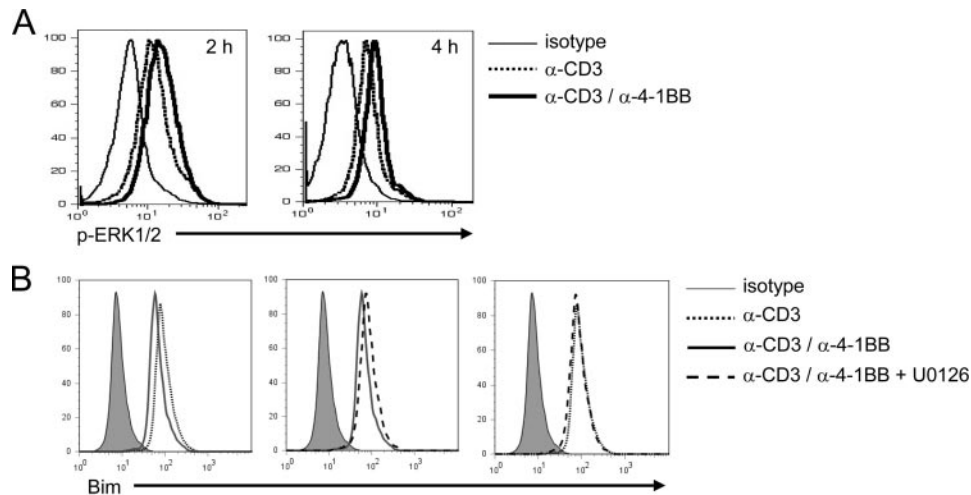


FIGURE 5. 4-1BB-dependent Bim modulation in anti-CD3 activated T cells is dependent on ERK activation. Purified WT T cells were stimulated with 1 $\mu g/ml$ anti-CD3 overnight to induce surface expression of 4-1BB. T cells were then rested for 24 h to reduce TCR-induced ERK levels and then were cultured in the absence or presence of plate-bound anti-4-1BB (10 $\mu g/ml$). *A*, Phospho-ERK levels in unstimulated vs anti-4-1BB stimulated T cells was analyzed at 2 and 4 h post-4-1BB stimulation using phospho-ERK1/2-specific Abs (pT202/Y204). *B*, Where indicated, cells were preincubated with the ERK inhibitor, U0126 for 30 min before anti-4-1BB stimulation and Bim levels monitored at 24 h by flow cytometry, filled histograms represent isotype control. These data are representative of two similar experiments.

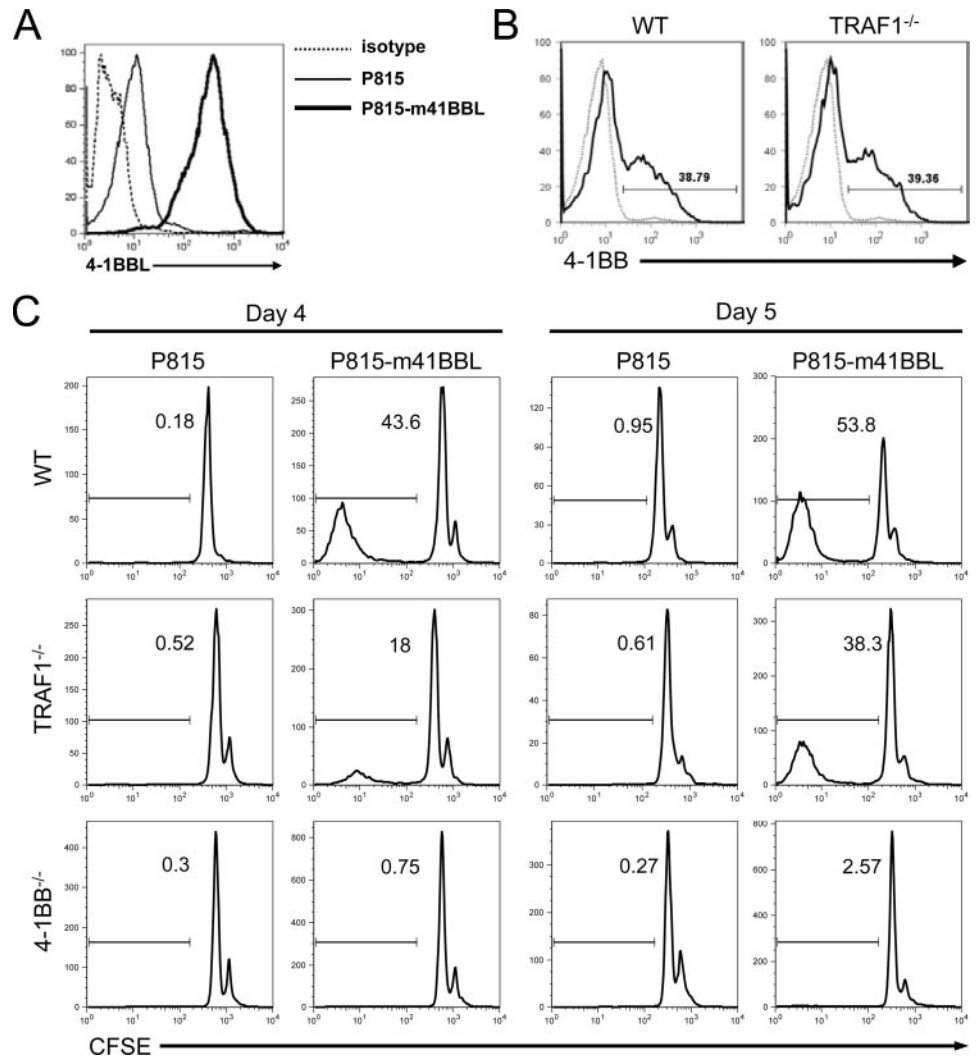


FIGURE 6. TRAF1 enhances cell division in a 4-1BB-costimulated alloresponse. *A*, P815 cells (H-2^d) were transfected with murine 4-1BBL and stained with anti-4-1BBL (TKS-1) or isotype control as indicated. *B*, WT or TRAF1-deficient T cells were analyzed for up-regulation of 4-1BB expression at 48 h following incubation with P815 cells. Dotted lines represent isotype control staining. *C*, T cells purified from WT, TRAF1^{-/-}, or 4-1BB^{-/-} B6 (H-2^b) mouse spleens were labeled with CFSE and 10⁶ labeled T cells were cultured with 10⁵ irradiated P815 or P815-4-1BBL cells for 4–5 days of culture and analyzed for CFSE expression. Plots are gated on live CD8 T cells. These data are representative of three similar experiments.

anti-CD3 in the presence or absence of immobilized 4-1BBL (11). Stimulation of WT T cells with anti-CD3 alone induced minimal IL-2 production, whereas coimmobilization of anti-CD3 and 4-1BBL resulted in IL-2 production by the T cells (Fig. 4*A*, left panel). TRAF1^{-/-} T cells showed a higher response to anti-CD3 alone, as reported previously (26), but failed to show significant augmentation with addition of 4-1BBL (Fig. 4*A*, middle panel). Both WT and TRAF1^{-/-} T cells responded similarly to anti-CD3 plus anti-CD28, resulting in significantly higher responses than observed with anti-CD3 alone (Fig. 4*A*, right panel). The finding that anti-CD3/anti-CD28 costimulation results in higher IL-2 production than anti-CD3/4-1BBL stimulation argues that the failure of TRAF1-deficient cells to show augmented IL-2 production in response to 4-1BBL is not due to the IL-2 production in the cultures reaching a maximum. These data suggest that TRAF1 is required for optimal 4-1BB-mediated costimulation of IL-2 production. We also examined the effect of 4-1BB on cell division using CFSE-labeled T cells (Fig. 4*B*). For WT T cells, there was minimal cell division in response to anti-CD3 alone, whereas costimulation with anti-CD3 plus anti-4-1BB induced cell division in 28% of the cells by 48 h. As was the case for IL-2 production, TRAF1-deficient T cells had a higher response to anti-CD3 alone compared with WT T cells and failed to increase their rate of division in response to 4-1BB.

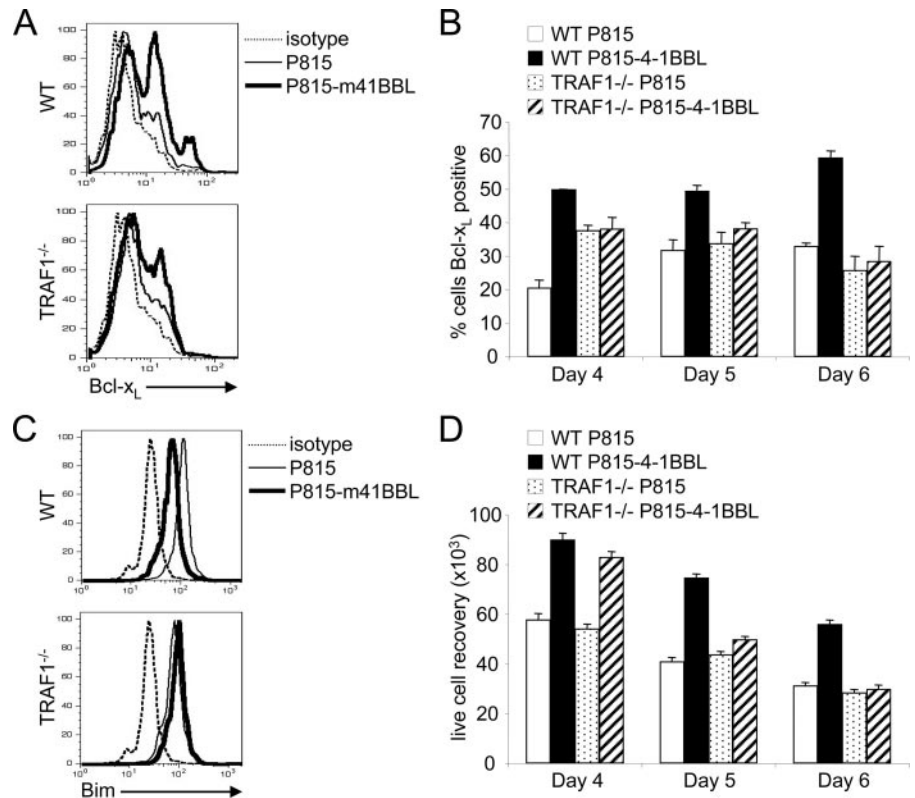
Role of ERK in Bim down-modulation following anti-CD3 and 4-1BB signaling

To determine whether activated ERK plays a role in Bim modulation downstream of 4-1BB engagement in this model, we first used anti-CD3 to induce 4-1BB expression on the T cells. Twenty-four hours following anti-CD3 stimulation, we used plate-bound anti-4-1BB Ab and monitored ERK activation and Bim modulation (Fig. 5, *A* and *B*). 4-1BB ligation resulted in increases in the level of activated ERK in anti-CD3 preactivated T cells in as little as 2 h (Fig. 5*A*), consistent with the results in the OT-I model (Fig. 3*B*). Furthermore, inhibition of ERK activation by pretreating the anti-CD3-activated T cells with the inhibitor U1026 30 min before addition of anti-4-1BB resulted in increased levels of Bim in the T cells (Fig. 5*B*). These data confirm in a second model, that 4-1BB engagement leads to ERK-mediated Bim down-modulation.

TRAF1^{-/-} T cells exhibit impaired proliferative capacity during an alloresponse in vitro

To extend the results observed in OT-I or anti-CD3-activated T cells to another model, we took advantage of the alloresponse of H-2^b T cells to the H-2^d expressing cell line P815. Irradiated P815 cells, with or without transfected 4-1BBL (Fig. 6*A*), were used to stimulate purified CFSE-labeled WT or TRAF1^{-/-} allogeneic T cells. By 48 h after allostimulation, the WT and TRAF1^{-/-} T cells

FIGURE 7. TRAF1 promotes T cell survival in a 4-1BBL-costimulated alloresponse by reciprocally regulating Bcl-x_L and Bim levels. T cells (H-2^b) were purified from WT and TRAF1^{-/-} mice and 10⁶ cells were cultured with 10⁵ irradiated P815 or P815-m4-1BBL (H-2^d) for 4–6 days. *A*, Representative intracellular staining for Bcl-x_L in WT and TRAF1^{-/-} T cells following 5-days incubation with P815 or P815-4-1BBL. *B*, Kinetics of Bcl-x_L expression in the T cells, expressed as % staining following stimulation with P815 or P815-4-1BBL was determined relative to isotype control staining. *C*, Bim expression in WT or TRAF1^{-/-} T cells following 5 days of culture with allostimulators. Results from *A–C* are gated on live CD8 T cells. *D*, Enumeration of live cells in the cultures based on Trypan blue exclusion. Data are representative of two similar experiments.



showed similar levels of 4-1BB on their surface (Fig. 6*B*). The failure of 4-1BB^{-/-} T cells to proliferate at all time points in response to P815-4-1BBL cells under these culture conditions, confirms the complete dependence of this model on the interaction of 4-1BBL with its receptor 4-1BB (Fig. 6*C*). Furthermore, responses of both WT and TRAF1-deficient T cells were completely dependent on costimulation. At day 4 of culture, TRAF1^{-/-} CD8 T cells showed a greater than 2-fold defect in division, when compared with WT T cells and a 35% defect by day 5 (Fig. 6*C*). These observations imply that in the absence of TRAF1 in an allostimulation model, 4-1BB-dependent signals are still able to costimulate proliferation of T cells, but TRAF1 contributes quantitatively and kinetically to this effect.

TRAF1 promotes T cell survival in a 4-1BBL-costimulated alloresponse by reciprocally regulating Bcl-x_L and Bim levels

Previous studies have shown that 4-1BB up-regulates Bcl-x_L through an NF-κB-dependent mechanism (16), but the effect of TRAF1 on this pathway has not been determined. Fig. 7, *A* and *B*, shows that during an alloresponse to P815-4-1BBL, WT T cells up-regulate Bcl-x_L in a higher proportion of cells than TRAF1-deficient T cells. The greatest effects of TRAF1 were observed at day 6, when ~60% of WT T cells express Bcl-x_L compared with 30% of TRAF1-deficient T cells (Fig. 7*B*). Bim levels were found to increase during stimulation with P815 cells and levels were reduced by 4-1BBL costimulation when T cells express TRAF1 but remain at higher levels in its absence (Fig. 7*C*). We also recovered an increased absolute number of viable cells at days 5–6 from cultures of WT T cells compared with cultures of TRAF1-deficient T cells when 4-1BBL was present on the stimulators (Fig. 7*D*). Thus, the presence of TRAF1 during 4-1BBL-mediated costimulation leads to increased Bcl-x_L and decreased Bim levels with a concomitant increase in T cell recovery from the cultures.

Discussion

In this study, we demonstrate that TRAF1 is required in T cells for maximal responses to 4-1BB signaling. *In vivo*, the expansion of OT-I CD8 T cells in response to Ag and 4-1BB signaling is dependent on the presence of TRAF1 in the T cells. In the absence of TRAF1 or 4-1BB-mediated costimulation, the T cells divided but there was decreased recovery of the CD8 T cells, consistent with a role for TRAF1 in T cell survival. On preactivated T cells, ERK is rapidly activated upon 4-1BB signaling and its activation correlates with Bim down-modulation. T cells lacking TRAF1 fail to activate ERK following 4-1BB stimulation and show increased levels of Bim. Conversely, inhibition of ERK activation on WT T cells prevents 4-1BB-mediated Bim down-modulation. During CD8 T cell responses to allo-APC overexpressing 4-1BBL, the presence of TRAF1 in the T cells increased the kinetics of cell division and increased T cell survival by increasing the level of Bcl-x_L and decreasing the level of Bim in the T cells. These observations show that 4-1BB uses two distinct signaling mechanisms to maintain T cell survival, the NF-κB-dependent up-regulation of Bcl-x_L expression (16) and the activation of ERK leading to Bim down-modulation (this report). The studies presented here implicate TRAF1 in both these survival mechanisms.

In the OT-I model, the main effects of 4-1BB and TRAF1 were on cell survival, with no detectable defect in cell division in TRAF1^{-/-} T cells. In contrast, in an *in vitro* model in which P815 cells with or without transfected 4-1BBL were used as allostimulators, TRAF1 played a role in both division and survival of T cells. In the *in vitro* transfection model, 4-1BBL represents the major costimulatory molecule present. In contrast, with the OT-I model, other costimulators present on physiological APC may contribute to cell division, so that the predominant role of both 4-1BB and TRAF1 is on survival of the T cells. The present findings that

the major effect of 4-1BB and TRAF1 are on T cell survival *in vivo*, are consistent with previous studies demonstrating a role for 4-1BB/4-1BBL in the survival of CD8 T cells following initial contraction of a response (4, 8) and in the Ag-independent maintenance of CD8 T cell memory (10, 43).

In a previous study, we showed that TRAF1^{-/-} CD8 T cells showed decreased expansion in a polyclonal response to influenza nuclear protein *in vivo* (29). Similarly, in this report, OT-I T cells lacking TRAF1 showed decreased survival during Ag-dependent expansion *in vivo* (Figs. 1, 2, and 3). In contrast, in a reductionist model using anti-CD3 with highly purified T cells, an apparently negative effect of TRAF1 is observed, consistent with a previous report (26). The hyper-responsiveness of TRAF1-deficient T cells to Ag has also been observed with DO11.0 MHC class II-restricted TCR transgenic T cells in an allergic airway inflammation model (27). It is possible that TRAF1 has a negative role downstream of Ag receptor signaling in some models. Alternatively, it is possible that TRAF1-binding TNFRs participate in the positive selection of T cells and that thymocytes that have matured in the absence of TRAF1 signals may have compensated for lack of TRAF1 in other ways. Indeed TRAF2-deficient T cells are also hyper-responsive to anti-CD3 (11), despite the well-established role of TRAF2 in pro-survival signaling (28). Regardless of possible effects on TCR signaling, in the present report, we show that when TCR plus 4-1BB signals are present, the predominant role of TRAF1 is as a mediator of survival signals downstream of 4-1BB.

The role of TRAF1 has previously been explored downstream of other TNFR family members. T cells from TRAF1^{-/-} mice were shown to be hyper-responsive to TNF, attributed to enhanced signaling through TNFR2, as TNFRI was not detected on the activated T cells. However, because cells were treated with anti-CD3 before the TNF treatment, the interpretation of the data is complicated by the hyper-responsiveness of TRAF1^{-/-} T cells to anti-CD3. In contrast, TRAF1 expression enhances JNK activation in response to TNF (21) and downstream of the EBV-encoded receptor LMP (44). Similarly, TRAF1 enhances TRAF2-dependent activation of NF- κ B downstream of CD40 signaling (21, 24, 25). However, the stoichiometry of TRAF1 to TRAF2 is critical, as overexpression of TRAF1 can inhibit TNF- and CD40-mediated TRAF2-dependent signaling (23, 24). These findings have led to the suggestion that a mixed trimer of two TRAF2 to one TRAF1 may be optimal for signaling downstream of CD40 (24).

A TRAF1/TRAF2 heterocomplex has been demonstrated downstream of TNF signaling, and is required for recruitment of cellular inhibitors of apoptosis (45). In B cells and dendritic cells, TRAF1 appears to cooperate with TRAF2 to ensure maximal TRAF2-dependent signaling. Furthermore, TRAF1 was found to cause dissociation of TRAF2 from lipid rafts and prevent the degradation of TRAF2 (21, 25). However, paradoxically, TRAF2 localization to lipid rafts is critical for its signaling function, leading to the suggestion that TRAF1 is important in recycling of TRAF2 for sequential signaling (21, 25). In the present study, we also found that TRAF2 levels are lower following 4-1BB signaling in TRAF1-deficient as compared with TRAF1-sufficient cells. Thus, it is possible that TRAF1 effects on T cell survival are indirect, via the maintenance of TRAF2 levels.

Previous results have shown that Bcl-x_L induction downstream of 4-1BB is dependent on NF- κ B activation. The finding that Bcl-x_L levels are reduced in TRAF1-deficient T cells is, therefore, consistent with TRAF1 contributing to TRAF2-mediated NF- κ B activation, despite the fact that TRAF1 lacks the E3 ubiquitin ligase domain required for direct activation of the NF- κ B pathway downstream of TNFRs.

The present study identifies ERK activation as an early signaling event downstream of 4-1BB signaling that is critical for the TRAF1-dependent regulation of Bim levels in activated T cells. ERK activation downstream of TNFRI requires RIP1 and TRAF2 (46), however, to our knowledge, the requirement for TRAF1 for ERK activation downstream of a specific TNFR family member has not been recognized to date. Whether the effects of TRAF1 on 4-1BB signaling are simply due to effects on TRAF2 or whether TRAF1 recruits distinct signaling molecules to the 4-1BB signalosome remains to be determined. However, the fact that removal of the N-terminal 163 amino acids of TRAF1 converts it from a modulator to a generalized inhibitor of NF- κ B activation (47), suggests that the N-terminal portion of TRAF1 may indeed have signaling functions independently of TRAF2. In primary T cells, the absence of TRAF2 completely abrogates 4-1BB-mediated costimulation (11). Thus, understanding the role of TRAF1 in survival signaling independently of TRAF2 will likely require the identification of TRAF1 binding proteins that link TRAF1 to downstream signals.

In sum, in this report, we show that TRAF1 is required for maximal Bcl-x_L induction and for ERK-dependent Bim down-modulation following 4-1BB stimulation on T cells. This effect of TRAF1 on increasing the ratio of Bcl-x_L to Bim correlates with increased recovery of TRAF1-sufficient compared with TRAF1-deficient T cells *in vitro* and *in vivo*. These studies identify TRAF1, ERK, and Bim down-regulation as a critical signaling axis leading to 4-1BB-mediated survival signaling in CD8 T cells.

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Disclosures

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