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TRAF1 phosphorylation on Serine 139 modulates NF-κB activity downstream of 4-1BB in T cells

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ABSTRACT

The Tumour Necrosis Factor (TNF) Receptor-associated factor-1 (TRAF1) adaptor protein is a key component in initiating intracellular signalling pathways downstream of TNF receptors (TNFR). More importantly, TRAF1 has a pattern of expression restricted primarily to lymphoid cells and plays an important role in lymphocyte survival. TRAF1 has been shown to be phosphorylated on Serine 139, consequently inhibiting NF-κB activation downstream of TNFR2 when expressed in HeLa cells. We have previously demonstrated that TRAF1 cooperates with the TNFR family member 4-1BB to mediate signalling in T cells. However, the impact of TRAF1 phosphorylation on events downstream of 4-1BB in T cells remained to be defined. Using a proteomics approach we demonstrate that TANK-binding kinase 1 (TBK1) preferentially associates with the TRAF1 Serine 139 to Alanine (S139A) mutant. TBK1 is a kinase that functions upstream of NIK and IKK in the activation of the NF-κB pathway. When TRAF1-deficient CD8 T cells were reconstituted with the TRAF1 S139A mutant, we observed more sustained levels of IκBα degradation in response to 4-1BB stimulation in contrast to cells expressing either TRAF1 wild-type or TRAF1 S139D phosphomimetic mutant. Together, these findings define the importance of the basal phosphorylation state of the TRAF1 Serine 139 residue in coordinating signalling events downstream of 4-1BB in primary T cells.

1. Introduction

Tumour Necrosis Factor Receptor (TNFR) family members play a role in cell survival and cell death. Among these receptors, CD27, CD30, OX40, 4-1BB, HVEM and GITR are co-stimulatory molecules that mediate their effects on T cells [1,2]. The signals provided by co-stimulatory molecules promote more sustained interactions of T cell receptors (TCR) with their cognate Major Histocompatibility Complex (MHC):peptide complexes, increase cell division, survival, and induce effector functions. TNFR signalling is dependent on the recruitment of TNFR-associated factor (TRAF) adaptor molecules to their cytoplasmic tails which triggers the activation of Erk, JNK, p38 and NF-κB pathways leading to cell survival and secretion of cytokines [3–6].

TRAF proteins have in common a conserved TRAF domain in the C-terminus responsible for association with TNFR cytoplasmic tails as well as for homotypic and heterotypic oligomerization. The N-terminal region of most TRAF proteins contain Zn fingers and a RING finger responsible for signalling through the stress activated protein kinase and NF-κB signalling pathways, respectively, with the exception of TRAF1 [7,8]. Additionally, TRAF1 has the most restricted expression pattern among the TRAF family members, found almost exclusively in lymphocytes, dendritic cells and certain epithelia [9–11]. TRAF1 cooperates with TRAF2 to activate the NF-κB and JNK pathways downstream of the CD40 receptor in B cells [12] and dendritic cells [13]. Additionally, TRAF1 expression was also shown to inhibit TNFR2-induced TRAF2 degradation, and enhance NF-κB activation in response to TNFR2 [14]. We have shown that TRAF1 is required downstream of 4-1BB to downmodulate the levels of the pro-apoptotic molecule Bim and enhance survival of CD8 T cells [5,6].

Interestingly, TRAF1 has been shown to be phosphorylated on Serine 139 by PKN1 (Serine 146on human TRAF1) when overexpressed in HeLa cells, consequently attenuating IKK/NF-κB and JNK activation downstream of TNFR2 [15]. However, the impact of TRAF1 phosphorylation on the recruitment of intracellular mediators and the induction of signalling pathways in response to 4-1BB co-stimulation in T cells remains to be defined. Using a systems biology approach and a TRAF1 Serine 139 to Alanine (S139A) mutant, we identified TANK-binding kinase 1 (TBK1), an

Abbreviations: TNFR, Tumour Necrosis Factor Receptor; TCR, T cell receptor; MHC, Major Histocompatibility Complex; TRAF, TNFR-associated factor; TBK1, TANK-binding kinase 1; WT, wildtype; OVA, ovalbumin.

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IκB kinase (IKK)-activating kinase [16], to preferentially associate with the TRAF1 signalling complex in the absence of phosphorylation on Ser139. In addition, the absence of TRAF1 from T cells leads to a reduction in TBK1 recruitment to the 4-1BB signalling complex upon receptor triggering. More importantly, TRAF1-deficient T cells reconstituted with the TRAF1 S139A mutant showed the highest levels of sustained IκBα degradation in contrast to cells reconstituted with the wild-type (WT) form of TRAF1 or a TRAF1 phospho-mimetic mutant (S139D). These findings highlight the importance of TRAF1 phosphorylation on Serine 139 in coordinating intracellular signalling events in response to 4-1BB stimulation in primary T cells.

2. Materials and methods

2.1. Mice

OT-I mice [17] were obtained from the Jackson Laboratories. OT-I TRAF1$^{-/-}$ mice have been described [6]. OT-I transgene expression was assayed using anti-Vα2 and anti-Vβ5.1 (eBiosciences, San Diego, CA, USA). Mice were maintained under SPF conditions in sterile microisolator caging. Mice studies were performed in accordance with the regulations of the Canadian Council on animal care.

2.2. Lymphocyte isolation and stimulation

Total splenocytes from OT-I WT and TRAF1$^{-/-}$ mice were incubated with 0.1 μg/ml of SIINFEKL peptide for 18 h, excess peptide was washed away and cells were rested for 24 h. At day 3 post activation, live CD8 T cells were isolated using Lympholyte-M density separation medium (Cedarlane, Burlington, ON, Canada). Purified CD8 OT-I cells were then stimulated with 10 μg/ml agonistic anti-4-1BB antibody (clone 3H3) or control rat IgG antibody (Sigma, Oakville, ON, Canada). Naïve CD8 T cells from OT-I mice spleens were isolated by negative depletion using magnetic bead separation (Easy Sep, StemCell, Vancouver, BC, Canada), purity was >90%. OT-I cells (10^6 cells/ml) were stimulated with 1 μg/ml plate bound anti-CD3ε (clone 145-2C11) for 24 h.

2.3. Retroviral transduction

Retroviral particles were produced in 293T cells (ATCC, Manassas, VA, USA) co-transfected with 10 μg of pMSCV-IRES-GFP vector (pMIG) expressing TRAF1 (WT, S139D and S139A) and 10 μg pCL-Eco retrovirus packaging vector (Imgenex, San Diego, CA, USA) using Poly(ethyleneimine) solution (Sigma). Viral supernatant were collected 48 h post-transfection, and diluted with T cell media at a 1:1 ratio. OT-I cells were infected at 37 °C at 2200 rpm in a tabletop centrifuge for 90 min at day 0 and 1 of activation. 48 h after infection GFP$^+$ cells were FACS sorted and expanded in 20 ng/ml recombinant human IL-15 (R&D Systems, Minneapolis, MN, USA) for 8 to 14 days. For Jurkat E6.1 infection, 293T cells were co-transfected with 10 μg of a vector expressing the Vesicular Stomatitis Virus glycoprotein.

2.4. Flow cytometry

Cells were surface-stained with anti-CD8α–PerCP (BD Pharmingen, Mississauga, ON, Canada), biotinylated anti-4-1BB and streptavidin-APC (eBioscience). Samples were acquired and analyzed using a FACSCalibur (BD Biosciences, Mountain View, CA) and FlowJo software (TreeStar Inc., Ashland, OR, USA), respectively. OT-I TRAF1$^{-/-}$ cells transduced with retroviral constructs were sorted on a FACSAria sorter (BD Biosciences).

2.5. Immunoprecipitation and Western blot analysis

After stimulation with anti-4-1BB or rat IgG control, cells were washed in cold PBS, lysed in Tris buffered saline containing 0.4 mM EDTA, 1% NP40, and the complete protease inhibitors cocktail (Roche, Mississauga, ON, Canada), immunoprecipitated using protein G-Sepharose beads (GE Healthcare Life Sciences, Baie d'Urfé, QC, Canada) coupled to 1 μg anti-4-1BB or anti-TRAF1 antibodies and subjected to SDS–PAGE, and then transferred to PVDF membranes (Pall Life Sciences, Mississauga, ON, Canada). Membranes were probed with antibodies specific for TRAF1 (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA), IκBα, TBκ1 (Cell Signaling Technology, Whitby, ON, Canada), 4-1BB (Alexis Biochemical, Burlington, ON, Canada) or β-actin (Sigma) and incubated with the HRP-conjugated anti-rabbit or anti-rat Ig antibody (Sigma). Signals were revealed with the Amersham ECL detection system (GE Healthcare Life Sciences) and digital images were acquired using the FujiFilm LAS-4000. Densitometry was performed using the Multi Gauge software (Fujifilm, Tokyo, Japan).

2.6. Mass spectrometry

Affinity-purified protein complexes were washed in 50 mM Tris pH 7.4 containing protease inhibitors. Proteins were eluted off protein G-Sepharose beads with 100 μl of 50 mM H_3PO_4, pH 1.8 on ice for 10 min, acidity verified (pH 2) and stored at −80 °C until tryptic digestion. Samples were processed using a solid-phase digest protocol, as previously described [18]. A QSTAR Elite QqTOF mass spectrometer equipped with a nanospray III ion source (AB Sciex, Concord, ON, Canada), and coupled to an Eksigent 1D + Nano LC (Dublin, CA, USA) was used. Tandem mass spectra were extracted, charge state deconvoluted and deisotoped in Analyst version 2.0. All MS/MS samples were analyzed using Mascot (Matrix Science, London, UK). Mascot was set up to search the subset of all murine proteins extracted from the ENSEMBL database. Searches were performed with carbamidomethyl (C) set as a fixed modification and deamidated (NQ), pyro-Glu (N-term Q), pyro-Glu (N-term E), phospho Tyr, and phospho Ser/Thr set as variable modifications. Trypsin was selected for enzyme digestion with up to 2 missed cleavages. QqTOF type fragmentation was selected with peptide mass tolerance set to 80 ppm, and fragment mass tolerance set to 0.15 Da. Scaffold (Proteome Software Inc., Portland, OR, USA) was used to validate MS/MS based peptide and protein identifications. Protein identifications were generally accepted if they could be established at greater than 95.0% probability and contained at least 1 identified peptide. Protein probabilities were assigned by the Protein Prophet algorithm.

3. Results

3.1. TBK1 associates with the TRAF1 complex in T cells

TRAF1 was shown to be phosphorylated on Serine 139 by PKN1 in the absence of activation, when overexpressed in HeLa cells [15]. We sought to determine the impact of this posttranslational event on signalling in T cells. Murine WT TRAF1 and a Ser 139 to Ala (S139A) mutant, which cannot be phosphorylated at Ser 139, were introduced into the Jurkat E6.1 human leukemic cell line by retroviral transduction with a vector that co-expresses the GFP. Transduction of the retroviral constructs expressing either the WT or S139A mutant into Jurkat cells was confirmed by flow cytometry based on GFP expression (Fig. 1A) and Western blotting analysis (data not shown). Murine TRAF1 was then immunoprecipitated and the complex was subjected to gel-free liquid chromatography coupled tandem mass spectrometry (LC-MS/MS) using solid phase

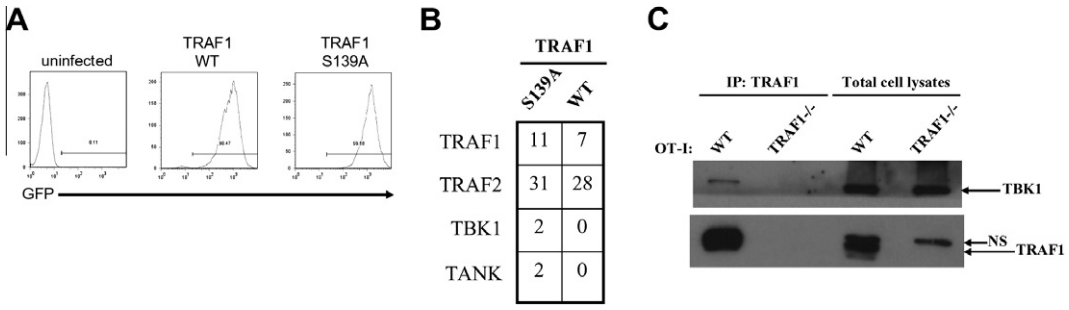

Fig. 1. TBK1 associates with TRAF1 in T cells. (A) Murine TRAF1 WT and S139A mutant were introduced into Jurkat E6.1 cells with a retroviral construct co-expressing GFP. FACS plots show >95% of cells are GFP$^+$. (B) Murine TRAF1 was immunoprecipitated and LC-MS/MS was then performed. The number of unique peptides for TRAF1, TRAF2, TBK1 and TANK identified by LC-MS/MS is shown. (C) Naïve CD8 T cells from OT-I mice (WT and TRAF1$^{-/-}$) were isolated, TRAF1 was then immunoprecipitated and the association of TRAF1 and TBK1 was analyzed by Western blot analysis. The expression of TBK1 and TRAF1 in total cell lysates from WT and TRAF1$^{-/-}$ cells is shown. NS, non-specific band.

tryptic digestion. TANK and TBK1, two molecules involved in NF-κB activation downstream of TNFRs family members [16,19,20], were identified to preferentially co-immunoprecipitate with the TRAF1 S139A mutant since we only observed TBK1 peptides from immunoprecipitates isolated from Jurkat cells expressing the TRAF1 S139A mutant (Fig. 1B). Of note, the amino acid sequences of human and murine TBK1 are 94% identical [16]. Primary CD8 T cells were then isolated from resting TCR transgenic (OT-I) mice, and TRAF1 was immunoprecipitated. Western blotting analysis confirmed the presence of TBK1 and TRAF1 within the same complex (Fig. 1C). As a control, immunoprecipitation was also performed on OT-I TRAF1$^{-/-}$ CD8 T cells.

3.2. Differential recruitment of TBK1 to TRAF1 phospho mutants

Due to the absence of a TRAF1 phospho-specific (Ser139) antibody, it is difficult to asses the phosphorylation state of TRAF1 in cells. Therefore, we also generated a Ser 139 to Asp mutant (S139D) as it was previously described that conversion of a Serine to Aspartate imitates phosphorylation [21]. Jurkat cells were stably transduced with retroviral vectors expressing the TRAF1 WT, S139D and S139A mutants and the levels of expression of the recombinant proteins was verified by Western blot analysis (Fig. 2A). In addition, we assessed the levels of protein expression of TBK1, which was similar in Jurkat cells expressing the different forms of TRAF1. Subsequently, we immunoprecipitated murine TRAF1 and determined the levels of endogenous TBK1 association with the TRAF1 signalling complex in resting cells. Densitometry analysis revealed that TBK1 preferentially associates with the TRAF1 signalling complex when it is not phosphorylated on Ser 139 (S139A mutant) (Fig. 2B). In contrast, the TRAF1 phospho-mimetic mutant (S139D) showed the lowest levels of TBK1 association. Interestingly, murine WT TRAF1 immunoprecipitated from Jurkat cells showed a ratio of TBK1 to TRAF1 that was intermediate to both mutants, which would suggest that a pool of TRAF1 might be phosphorylated within a cell (Fig. 2B). These findings demonstrate that the level of TBK1 recruitment to the TRAF1 signalling complex is dependent on the basal phosphorylation state of TRAF1.

3.3. Reduced TBK1 recruitment to the 4-1BB receptor in the absence of TRAF1

4-1BB is a TRAF1 binding receptor and its expression on the cell surface is induced upon activation through the TCR and co-stimulation [22]. Furthermore, we have previously demonstrated that 4-1BB and TRAF1 cooperate to induce downstream signalling in T cells [5]. To investigate the impact of the association of TRAF1 and TBK1 downstream of 4-1BB signalling, we used a TCR

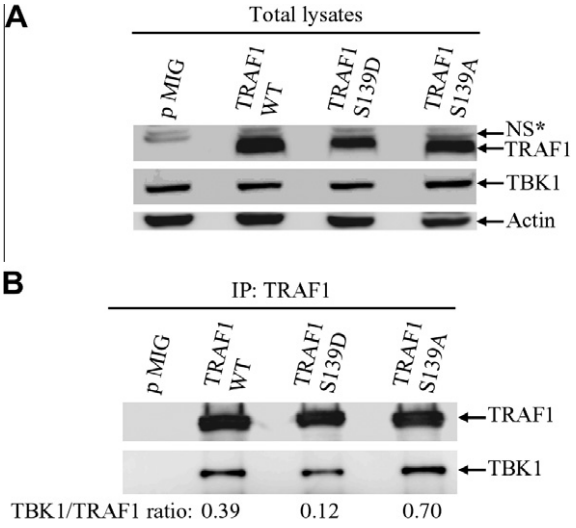

Fig. 2. TBK1 preferentially associates with the TRAF1 S139A mutant. (A) Jurkat cells were transduced with the empty retroviral vector (pMIG), or the vector co-expressing TRAF1 WT, TRAF1 S139A or TRAF1 S139D. The levels of expression of the different forms of TRAF1, TBK1 and actin were determined in total lysates by Western blot analysis. *NS, non-specific band. (B) TRAF1 was immunoprecipitated from Jurkat cells expressing TRAF1 WT, S139D or S139A and the levels of TBK1 associated with the TRAF1 complex was determined by Western blot. TBK1/TRAF1 ratios were determined by densitometry analysis. Representative results of three independent experiments.

transgenic model (OT-I) where the CD8 T cells have specificity to an ovalbumin (OVA) epitope (SIINFEKL) presented by the H-2K^b MHC class I molecule. OT-I WT or TRAF1$^{-/-}$ splenocytes were activated with the OVA peptide and at 48 h the majority of CD8 T cells from OT-I WT or TRAF1$^{-/-}$ cultures were found to express 4-1BB on the cell surface (Fig. 3A). Activated OT-I T cells were then stimulated with an agonistic anti-4-1BB antibody or a rat IgG control antibody for 20 min and the TRAF1 signalling complex was immunoprecipitated. As determined by Western blot analysis, the levels of TBK1 within the TRAF1 complex in OT-I WT cells are similar in the absence (rat IgG) or presence of 4-1BB stimulation (Fig. 3B). As a specificity control, immunoprecipitations were also performed on lysates from OT-I TRAF1$^{-/-}$ cells.

Subsequently, we immunoprecipitated the 4-1BB signalling complex from OVA peptide activated OT-I cells in the absence (rat IgG) or presence of 4-1BB stimulation from OT-I WT and TRAF1$^{-/-}$ cells and determined the levels of TBK1 within the 4-1BB complex. Western blot analysis revealed that stimulation through 4-1BB does not alter the levels of TBK1 associated with the 4-1BB signalling complex in OT-I WT cells (Fig. 3C). In addition,

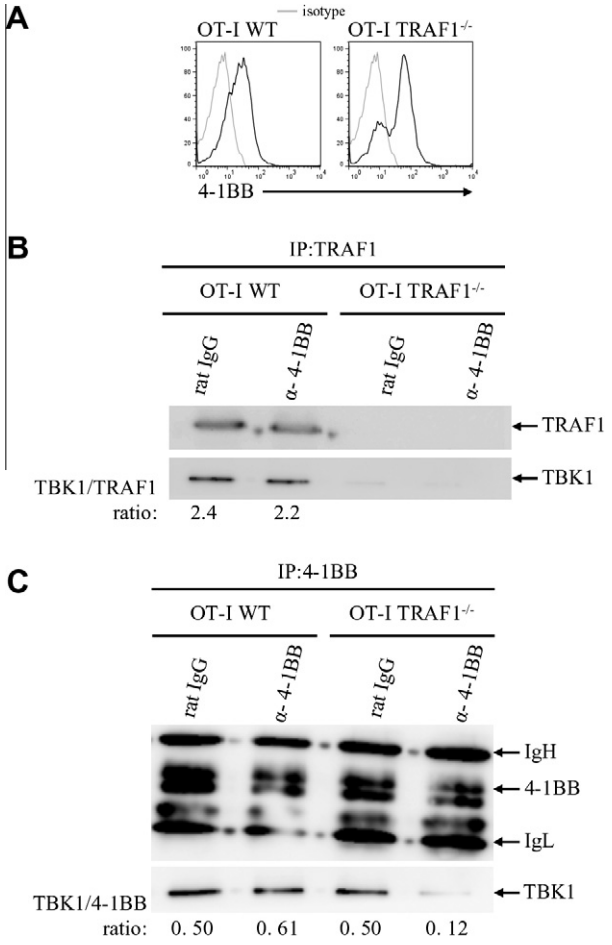

Fig. 3. Requirement of TRAF1 for the recruitment of TBK1 to the 4-1BB complex. OT-I splenocytes (WT and TRAF1$^{-/-}$) were stimulated with 0.1 μg/ml OVA peptide for 48 h. (A) Live CD8 T cells were isolated from the cultures at day 3 and 4-1BB surface expression was confirmed by flow cytometry. (B) Following the stimulation of cells for 20 min. with anti-4-1BB or control rat IgG, cells were lysed and the TRAF1 complex was immunoprecipitated. TRAF1 and TBK1 levels were determined by Western blot analysis. TBK1/TRAF1 ratios were determined by densitometry analysis. (C) Similarly to (B) cells were lysed after 4-1BB or rat IgG control stimulation and the 4-1BB complex was immunoprecipitated. 4-1BB and TBK1 levels were determined by Westen blot analysis. TBK1/4-1BB ratios were determined by densitometry analysis. Representative results of four independent experiments.

the levels of TBK1 associated with the 4-1BB complex were also similar in OT-I TRAF1$^{-/-}$ cells prior to 4-1BB stimulation (rat IgG control). However, when OT-I TRAF1$^{-/-}$ cells were stimulated with the 4-1BB agonistic antibody we observed 4-fold less TBK1 within the 4-1BB complex when compared to WT cells. These results demonstrate that TRAF1 plays a critical role in maintaining the proper ratio of signalling molecules recruited to 4-1BB in T cells upon receptor triggering.

3.4. Enhanced NF-κB activation with the TRAF1 S139A mutant downstream of 4-1BB signalling

TRAF1 phosphorylation on Ser139 by PKN1, has been shown to silence the activity of NF-κB signalling downstream of TNFR2 when overexpressed in HeLa cells [15]. Additionally, TBK1 is an upstream initiator of the NF-κB pathway downstream of TNFR signalling [16]. We sought to determine the impact of TRAF1 phosphorylation on NF-κB activation downstream of the 4-1BB receptor in CD8 T cells (OT-I). Initially, retroviral constructs expressing TRAF1 WT, S139D or S139A mutant were introduced into activated OT-I TRAF1$^{-/-}$ cells. Protein expression levels of TRAF1 WT, S139D and S139A in reconstituted OT-I TRAF1$^{-/-}$ cells were confirmed by Western blotting (Fig. 4A). OT-I cells were then stimulated with anti-CD3ε to induce 4-1BB cell surface expression, which was confirmed by flow cytometry (Fig. 4B). OT-I TRAF1$^{-/-}$ cells were also transduced with the empty vector expressing only the GFP tag as a control. Cells were then stimulated with the 4-1BB agonistic antibody or rat IgG control and the degradation of the NF-κB inhibitor, IκBα, was determined by Western blot analysis (Fig. 4C). Crosslinking of 4-1BB on OT-I cells expressing the TRAF1 S139A mutant demonstrated the most sustained NF-κB activation, based on the degradation of IκBα, whereas OT-I TRAF1$^{-/-}$ cells transduced with the empty vector showed the least sustained response. Interestingly, cells expressing the TRAF1 S139D mutant showed a small reduction in IκBα levels following 4-1BB stimulation (Fig. 4C). Collectively, these findings demonstrate that the presence and phosphorylation state of TRAF1 has an impact on the association of TBK1 with the 4-1BB signalling complex and NF-κB activation in T cells.

4. Discussion

Phosphorylation of TRAFs downstream of TNFRs has been previously described [23–25]. Murine TRAF1 was shown to be phosphorylated on Serine 139 by the kinase PKN1, a member of the PKC superfamily, which silenced IKK/NF-κB activity downstream of TNFR2 [15]. In this study, we report that substitution of the TRAF1 Serine 139 with an Alanine, which eliminates phosphorylation on this residue, leads to enhanced recruitment of TBK1 to the TRAF1 signalling complex. In addition, the presence of TRAF1 was required in T cells to ensure efficient recruitment of TBK1 to the 4-1BB complex upon receptor engagement. TBK1 is required to form a ternary complex with TRAF2 and TANK to mediate its activity, and represents an alternative way for TRAF-mediated activation of NF-κB downstream of TNFRs [16]. However, our findings demonstrate that the presence of TRAF1 seems to be essential downstream of 4-1BB. Indeed, when OT-I TRAF1$^{-/-}$ cells were stimulated with an agnostic 4-1BB antibody, we observed an important reduction in TBK1 levels within the 4-1BB receptor complex (Fig. 3C). Interestingly, we have previously shown that the absence of TRAF1 in OT-I cells leads to a reduction in the levels of TRAF2 following 4-1BB stimulation [5]. Similar observations have been reported by others regarding TRAF1-dependent stabilization of TRAF2 levels downstream of other TNFRs [12–14]. Thus, TRAF1 may regulate the levels of TBK1 within the 4-1BB receptor complex indirectly through the stabilisation of TRAF2 protein levels. However, the basal phosphorylation state of TRAF1 on Serine 139 is critical to maintain the proper ratio of signalling molecules downstream of 4-1BB in T cells.

TRAF1 was originally thought to be a negative regulator of TNF signalling, since TRAF1-deficient T cells were found to hyperproliferate in response to anti-CD3 stimulation [26]. In addition, given that TRAF1 lacks a RING finger domain present in other TRAF family members to mediate NF-κB activation, it resembled a dominant negative form of TRAF2. In contrast, our previous findings and other studies have demonstrated a positive regulatory role for TRAF1 in cells of the immune system [5,6,12,13,27]. The current study demonstrates that phosphorylation of TRAF1 modulates NF-κB activity by coordinating the recruitment of signalling molecules to the 4-1BB receptor in primary T cells. More recently, TRAF1 was shown to restrict the alternative NF-κB pathway but was required for the induction of the classical pathway in response to 4-1BB in T cells [28]. Phosphorylation of TRAF1 on Serine 139 could dictate which NF-κB pathway is induced downstream of

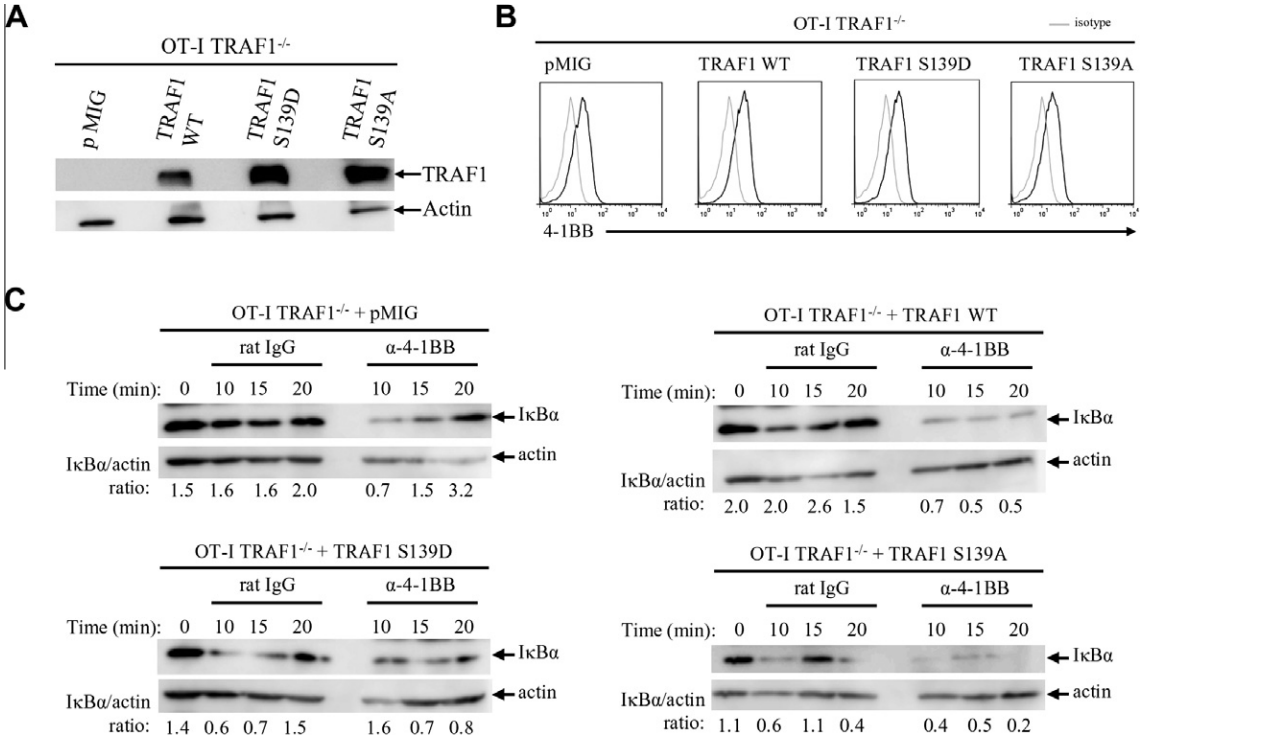

Fig. 4. Impact of TRAF1 Serine 139 mutants on NF-κB activation in response to 4-1BB stimulation. OT-I TRAF1$^{-/-}$ splenocytes were stimulated with 0.1 μg/ml OVA peptide and transduced with either the empty retroviral vector (pMIG) expressing the GFP alone or in parallel with the TRAF1 WT, TRAF1 S139D or TRAF1 S139A mutants. Live OT-I CD8 T cells expressing the TRAF1 variants were sorted based on GFP expression and expanded in IL-15 *in vitro*. (A) The levels of expression of the different forms of TRAF1 and actin were determined in total lysates by Western blot analysis. (B) Cells were restimulated with 1 μg/ml of anti-CD3ε for 24 h to induce 4-1BB surface expression which was verified by flow cytometry. (C) Cells were then stimulated for the indicated time point with anti-4-1BB or control rat IgG and the levels of IκBα and actin were determined by Western blot analysis. IκBα/actin ratios were determined by densitometry analysis. Representative results of three independent experiments.

4-1BB signalling. When OT-I TRAF1$^{-/-}$ cells expressed the S139A TRAF1 mutant, we consistently observed fluctuations in IκBα levels in the absence of 4-1BB stimulation (rat IgG control), in contrast to TRAF1-deficient cells or cells expressing WT TRAF1 (Fig. 4C). This demonstrates that the basal phosphorylation state of TRAF1 on Serine 139 may impact basal NF-κB activity in T cells in the absence of TNFR triggering, similarly to what was described when TRAF1 S139A was overexpressed in resting HeLa cells [15]. Furthermore, we consistently recovered significantly less OT-I cells expressing the TRAF1 S139D mutant in culture compared to TRAF1 WT or S139A expressing cells (data not shown), demonstrating the negative regulatory effects of TRAF1 phosphorylation on the maintenance of cells *in vitro*. However, it is clear that the absence of TRAF1 phosphorylation on Serine 139 is required for sustained NF-κB activation in response to 4-1BB signalling in T cells when compared with cells expressing the TRAF1 S139D phosphomimetic mutant or cells lacking TRAF1.

Collectively, the present observations provide a novel insight into the impact of TRAF1 phosphorylation in fine tuning intracellular events by coordinating the recruitment of signalling mediators involved in NF-κB activation following 4-1BB co-stimulation in T cells. We have previously demonstrated that TRAF1 was required for the maintenance of CD8 memory T cells *in vivo* and downstream of 4-1BB signalling [5,6]. The importance of TRAF1 phosphorylation on the maintenance of immunological memory will be investigated in future studies.

Acknowledgments

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