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RESEARCH ARTICLE

TRAF2 exerts opposing effects on basal and TNFα-induced activation of the classic IKK complex in hematopoietic cells in mice

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ABSTRACT

The role of TRAF2 and TRAF5 in TNFα-induced NF-κB activation has become complicated owing to the accumulation of conflicting data. Here, we report that 7-day-old TRAF2-knockout (KO) and TRAF2 TRAF5 double KO (TRAF2/5-DKO) mice exhibit enhanced canonical IκB kinase (IKK) and caspase-8 activation in spleen and liver, and that subsequent knockout of TNFα suppresses the basal activity of caspase-8, but not of IKK. In primary TRAF2 KO and TRAF2/5-DKO cells, TNFα-induced immediate IKK activation is impaired, whereas delayed IKK activation occurs normally; as such, owing to elevated basal and TNFα-induced delayed IKK activation, TNFα stimulation leads to significantly increased induction of a subset of NF-κB-dependent genes in these cells. In line with this, both TRAF2 KO and TRAF2/5-DKO mice succumb to a sublethal dose of TNFα owing to increased expression of NF-κB target genes, diarrhea and bradypnea. Notably, depletion of IAP1 and IAP2 (also known as BIRC2 and BIRC3, respectively) also results in elevated basal IKK activation that is independent of autocrine TNFα production and that impairs TNFα-induced immediate IKK activation. These data reveal that TRAF2, IAP1 and IAP2, but not TRAF5, cooperatively regulate basal and TNFα-induced immediate IKK activation.

KEY WORDS: TNFα, TRAF2, TRAF5, NF-κB, Apoptosis

INTRODUCTION

Members of the TNF superfamily regulate both the innate and adaptive immune responses by activating the canonical and noncanonical NF-κB pathways (Bonizzi and Karin, 2004; Hayden and Ghosh, 2008; Wajant and Scheurich, 2011). The majority of TNF-superfamily members activate the canonical NF-κB [e.g. involving NF-κB subunits p65 (also known as RelA) and p50 (encoded by *Nfkb1*)] pathway through the classic IκB kinase (IKK) complex (comprising IKKα, IKKβ and IKKγ/NEMO), leading to the expression of genes that modulate inflammation and innate immune response. In comparison, only a subgroup of TNF-superfamily members (e.g. CD40 ligand, B-cell-activating factor, and lymphotoxin α and lymphotoxin β) activate the noncanonical NF-κB [e.g. RelB and p52 (encoded by *Nfkb2*)] pathway through NF-κB-inducing kinase (NIK) and IKKα homodimers to promote the expression of genes that regulate adaptive immunity and lymphoid organ development (Bonizzi and Karin, 2004).

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TNFα (officially known as TNF) is a prototypical member of the TNF superfamily that only activates canonical NF-κB through its cognate receptor TNFR1 (also known as TNFRSF1A) (Hayden and Ghosh, 2008; Wajant and Scheurich, 2011). The current model for this signaling is that, upon TNFR1 ligation, TNFR-associated factor 2 (TRAF2) and TRAF5 redundantly catalyze polyubiquitylation of receptor-interacting protein 1 (RIP1; also known as RIPK1) through a K63 ubiquitin linkage at K377, and that the RIP1 K63 polyubiquitin chain then recruits the TAK1 and IKK complexes to trigger TAK1-mediated IKK activation (Chen and Sun, 2009; Wu et al., 2006). However, several recent studies suggest that TRAF2 lacks intrinsic E3 ligase activity and that it recruits inhibitor of apoptosis proteins 1 and 2 (IAP1 and IAP2; also known as BIRC2 and BIRC3, respectively) to promote RIP1 ubiquitylation (Mahoney et al., 2008; Varfolomeev et al., 2008; Vince et al., 2009; Yin et al., 2009). By contrast, a more recent study has revealed that sphingosine-1-phosphate (S1P) binds to the RING domain of TRAF2 and that this binding is required for TRAF2 E3 ligase activity (Alvarez et al., 2010).

TRAF2-knockout (TRAF2-KO) cells are defective in TNFα-induced JNK activation, but are only partially deficient in NF-κB activation (Yeh et al., 1997). Currently, it is believed that TRAF5 can mediate NF-κB activation in TRAF2-KO cells in response to TNFα stimulation, and that TRAF2 and TRAF5 double knockout (TRAF2/5-DKO) cells are completely defective in TNFα-induced RIP1 ubiquitylation and NF-κB activation (Hayden and Ghosh, 2008; Tada et al., 2001). Notably, RIP1 and NEMO are also ubiquitylated through a linear-linkage (aka at residue Met1; M1 linkage) by the HOIL-1L–HOIP complex, and such ubiquitylation has been shown to stabilize the TNFR1 complex to facilitate efficient NF-κB activation (Haas et al., 2009; Iwai and Tokunaga, 2009). Surprisingly, RIP1 expression itself has been reported to not be required for TNFα-induced NF-κB activation (Wong et al., 2010). By contrast, NIK accumulates, and the noncanonical NF-κB pathway is constitutively activated, in TRAF2-KO, TRAF3-KO and IAP1-IAP2-DKO cells, suggesting that TRAF2, TRAF3, IAP1 and IAP2 cooperatively target NIK for degradation in order to suppress noncanonical NF-κB in unstimulated cells (Habelhah, 2010; Hayden and Ghosh, 2008; Vince et al., 2009).

Notably, TRAF2-deficient macrophages produce large amounts of TNFα and nitric oxide (NO) in response to TNFα stimulation, suggesting that TRAF2 also suppresses certain aspects of the TNFα–TNFR1–NF-κB signaling axis (Nguyen et al., 1999). However, the mechanisms by which TRAF2 positively or negatively regulates TNFR1 signaling are still elusive. To clarify the roles of TRAF2 and TRAF5 in TNFα-induced NF-κB activation, we extensively analyzed the basal and TNFα-induced activation of the canonical NF-κB pathway at the levels of IKK activation kinetics, IκBα (also known as NFKBIA) degradation and NF-κB target gene expression in tissues and primary hematopoietic cells

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derived from TRAF2-KO, TRAF2/5-DKO, TNFα and TRAF2 (TNF/TRAF2)-DKO and TNFα, TRAF2 and TRAF5 (TNF/TRAF2/5) triple KO (TKO) mice. We report here that TRAF2, but not TRAF5, suppresses the basal activity of canonical NF-κB while playing an essential role in TNFα-induced immediate but not delayed IKK activation. Owing to this elevated basal and TNFα-induced delayed NF-κB activation, TNFα stimulation leads to significantly increased expression of a subgroup of NF-κB-dependent genes in TRAF2-KO and TRAF2/5-DKO primary cells. These results not only shed new light on the mechanisms by which TRAF2 negatively and/or positively regulates NF-κB activation depending on cellular context, but they also provide explanations for the conflicting conclusions that have been drawn based on analysis of IκBα degradation or NF-κB target gene expression.

RESULTS

TRAF2 suppresses the basal activity of the IKK complex in 7-day-old spleen and liver

To define the role of TRAF2 and TRAF5 in TNFα-induced NF-κB activation, we generated TRAF2-KO and TRAF2/5-DKO mice by crossing TRAF2$^{-/+}$ and TRAF5$^{-/-}$ mice. To assess the role of TRAF2 and TRAF5 in basal NF-κB activation, we first performed an IKK immunokinase assay to examine the basal IKK activity in various tissues derived from wild-type and TRAF2-KO mice. To rule out any possible contribution of IKKα homodimer in kinase assays, the IKK complex was immunoprecipitated using an antibody against IKKγ, and then the immunocomplex-bound beads were extensively washed with buffer containing 1% Triton X-100 and 0.5 M NaCl. As shown in Fig. 1A, the basal activity of the classic IKK complex was clearly enhanced in spleens of 7-day-

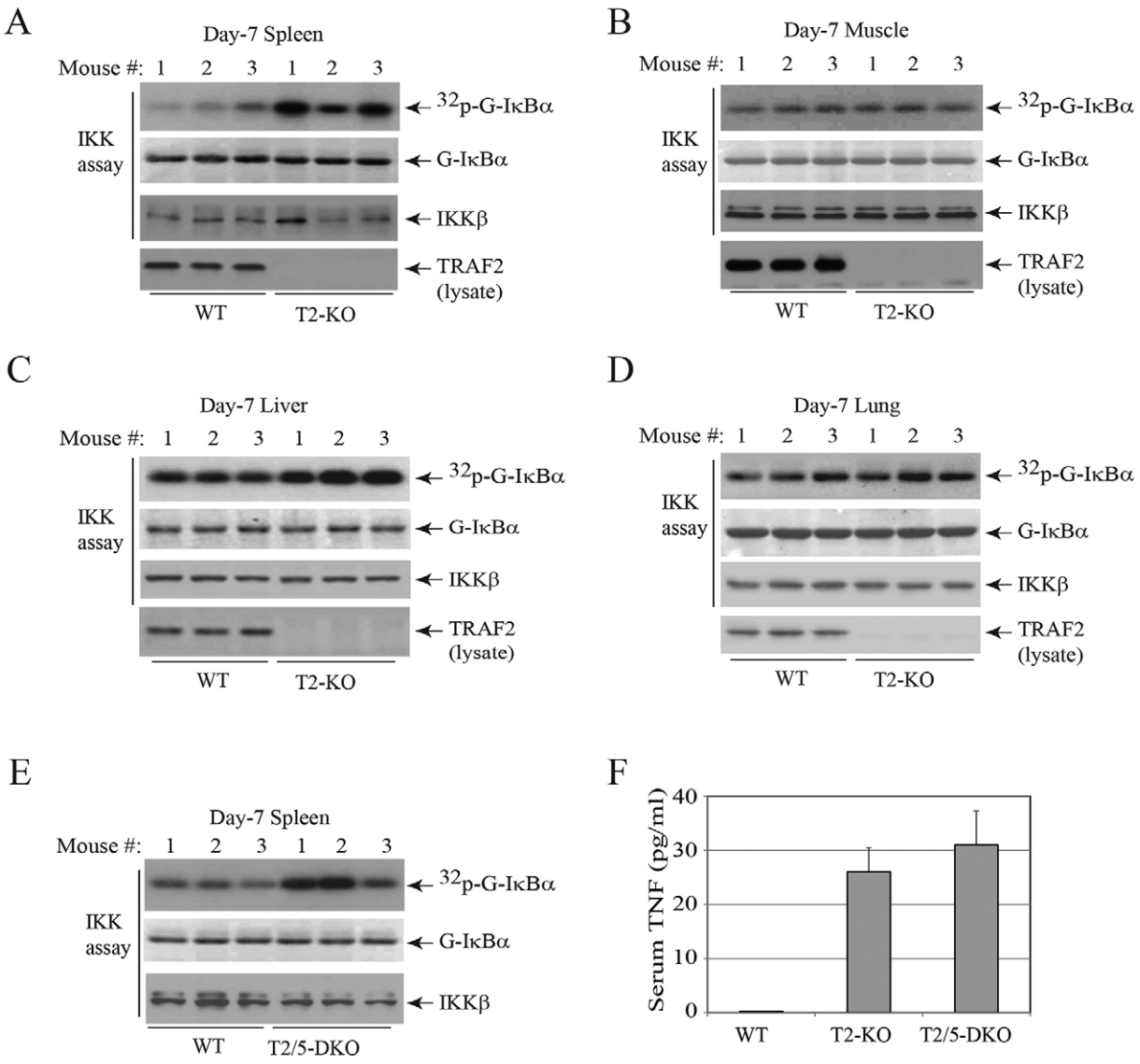

Fig. 1. TRAF2 suppresses the basal activity of the classic IKK complex in spleen and liver. (A–D) Total protein samples were extracted from the spleens, muscles, livers and lungs of 7-day-old wild-type (WT) and TRAF2-KO (T2-KO) mice, and then the classic IKK complex was immunoprecipitated with an antibody against IKKγ. After extensive washing, the IKK complex was subjected to *in vitro* kinase assays using GST–IκBα residues 1–55 (G-IκBα) as substrate. The reaction mixtures were separated by SDS–PAGE, transferred onto nitrocellulose membranes and exposed to X-ray film for 4–6 h (^{32}p-G-IκBα, ^{32}P-labeled GST–IκBα). The same membranes were stained with Ponceau S (to detect G-IκBα) and blotted for IKKβ. (E) The spleen was removed from 7-day-old WT and TRAF2/5-DKO mice, and IKK activity was determined by kinase assays as described above. (F) Serum TNF concentrations were determined by ELISA in TRAF2-KO and TRAF2/5-DKO (T2/5-DKO) mice and their littermate controls (mean±s.d., *n*=3).

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old TRAF2-KO mice compared to that of wild-type counterparts. The basal IKK activity was also slightly elevated in TRAF2-KO livers, but not in the lungs or muscles (Fig. 1B–D). In TRAF2/5-DKO mice, similar results were observed with respect to basal IKK activity in various tissues (Fig. 1E) (data not shown). TRAF2-KO mice have elevated basal serum TNFα levels (Nguyen et al., 1999). Analysis of serum TNFα levels also revealed that both TRAF2-KO and TRAF2/5-DKO mice had elevated levels of serum TNFα to a similar degree, whereas the serum TNFα levels in wild-type mice was undetectable (Fig. 1F). These data demonstrate that TRAF2, but not TRAF5, negatively regulates basal activity of the canonical IKK complex, at least at the early developmental stage of spleen and liver.

TRAF2-deficient spleens exhibit elevated expression of NF-κB-dependent genes

To further examine canonical NF-κB activation in TRAF2-KO and TRAF2/5-DKO tissues, we analyzed the expression of well-known NF-κB-regulated genes by performing real-time reverse transcription (RT)-PCR. Consistent with the elevated basal IKK activity, the expression levels of IAP2, TNFα, RANTES, IL-6 and IP-10 were significantly higher in 7-day-old TRAF2-KO and TRAF2/5-DKO spleens when compared to that of wild-type counterparts (Fig. 2A). Notably, these genes were also slightly upregulated in TRAF2-KO and TRAF2/5-DKO muscles compared to wild-type controls; however, the differences were not statistically significant (Fig. 2B). IKK-dependent phosphorylation and nuclear accumulation of p65/RelA and NIK-dependent p100 (encoded by *Nfkb2*) processing to p52 are hallmarks of canonical and noncanonical NF-κB activation, respectively. Therefore, to better understand the possible mechanisms for variable expression of NF-κB target genes in TRAF2-KO spleen and muscle, we examined IKK and p65 phosphorylation, NIK expression and processing of p100 in these tissues. Interestingly, p100 was constitutively processed to p52 in all TRAF2-KO tissues tested; however, the expression of NIK and phosphorylation of IKK and p65 was clearly higher in spleen than in muscle, and IKK and p65 phosphorylation was also slightly higher in TRAF2-KO spleen than in wild-type counterparts (Fig. 2C,D). Consistently, more NF-κB (p65) accumulated in the nuclei of TRAF2-KO spleen compared to that of wild-type counterparts (Fig. 2E). By contrast, JNK activity was unaltered in TRAF2-KO spleen compared to wild-type spleen (Fig. 2F). Western blot analysis of other TRAF2-regulated proteins, such as FLIP (also known as CFLAR), IAP1 and IAP2, and caspase-8, revealed that the expression of these proteins were clearly lower in muscle than in spleen. Interestingly, although IAP1 and IAP2 mRNA expression was higher in TRAF2-KO spleens, their protein levels were lower compared to that in wild-type counterparts (Fig. 2A,D). It is known that TRAF2 forms a complex with IAP1 and IAP2, and that IAP1 and IAP2 become unstable and undergo ubiquitylation-dependent degradation in the absence of TRAF2 (Csomos et al., 2009; Vince et al., 2009); thus, the decrease in IAP1 and IAP2 protein level in TRAF2-KO tissues is most likely due to post-translational degradation. In addition, the partially processed form of caspase-8 (the p46 fragment, denoted p46casp-8) was readily detectable in the liver and spleen of TRAF2-KO mice. These data suggest that the higher basal activity of canonical NF-κB in TRAF2-KO spleens contributes to elevated expression of NF-κB target genes and that caspase-8 is at least partially activated in TRAF2-deficient spleen and liver.

TRAF2 suppresses basal IKK activity in primary thymic T cells

To further examine the role of TRAF2 and TRAF5 in basal and inducible NF-κB activation, thymic T cells were isolated from 7-day-old wild-type, TRAF2-KO and TRAF2/5-DKO mice, and cultured for 4 h prior to stimulation with TNFα. As shown in Fig. 3A,B, TRAF2-KO and TRAF2/5-DKO thymic T cells also exhibited elevated basal IKK activity, and stimulation of these cells with TNFα further but weakly increased IKK activity. Analysis of NF-κB target genes in these cells revealed that RANTES and IP-10 (also known as CCL5 and CXCL10, respectively) expression was significantly elevated in TRAF2-KO and TRAF2/5-DKO cells following TNFα stimulation, whereas IL-6 expression was almost completely impaired (Fig. 3C). In contrast, western blot analysis revealed that IκBα degradation was kinetically delayed and incomplete in TRAF2-KO and TRAF2/5-DKO cells (Fig. 3D). These data suggest that TRAF2 also suppresses the basal activity of the classic IKK complex in primary cells, and that TNFα can induce canonical NF-κB activation in both TRAF2-KO and TRAF2/5-DKO primary cells to a similar extent.

TRAF2 deficiency results in elevated canonical NF-κB activation independent of TNFα production

TRAF2-KO mice exhibit elevated serum TNFα levels (Nguyen et al., 1999), which could cause the elevated IKK activity observed in the spleen and liver. To test this possibility, we generated TNF/TRAF2-DKO mice and then analyzed IKK activity. As shown in Fig. 4A, basal IKK activity was slightly reduced in TNF/TRAF2-DKO spleens compared to TRAF2-KO counterparts, but it was still clearly higher than that in TNF-KO spleens, and knockout of TNFα (TNF-KO mice) had no effect on NIK protein level. In line with this, expression of IAP2, RANTES and IP-10 in TNF/TRAF2-DKO spleens was reduced but not suppressed to the baseline levels seen in TNF-KO spleens (Fig. 4B). Notably, knockout of TNFα in the TRAF2-KO background restored IAP1 and IAP2 protein levels, and inhibited caspase-8 processing to p46casp-8 in the spleen (Fig. 4C); however, the knockout had no effect on constitutive p100 processing (Fig. 4D). These data suggest that the elevated IKK activation in TRAF2-KO spleens is partially due to the effect of autocrine TNFα and that a TNFα-independent mechanism also contributes to this event.

TRAF2 is essential for the immediate phase of IKK activation

The relatively longer survival of TNF/TRAF2-DKO and TNF/TRAF2/5-TKO mice allowed us to prepare a sufficient amount of bone-marrow-derived macrophages (BMDMs) to analyze the kinetics of TNFα-induced IKK activation. As shown in Fig. 5A,B, TNFα elicited immediate and strong IKK activation within 5 min of stimulation in TNF-KO BMDMs, which was completely impaired in TNF/TRAF2-DKO and TNF/TRAF2/5-TKO BMDMs. Notably, these DKO and TKO BMDMs also exhibited elevated basal IKK activity, and TNFα stimulation still induced delayed IKK activation (~10 min post stimulation). As expected, TNFα stimulation resulted in significantly increased expression of RANTES and IP-10, but not of IL-6, in these DKO and TKO BMDMs (Fig. 5C). Similarly, depletion of IAP1 and IAP2 with Smac mimetic also led to a clear increase in basal IKK activity and significantly but not completely impaired TNFα-induced delayed IKK activation in wild-type and TNF-KO BMDMs (Fig. 5D). These data suggest that TRAF2, IAP1 and IAP2 cooperatively suppress basal IKK activity in unstimulated cells and play essential roles in TNFα-induced immediate and robust IKK activation.

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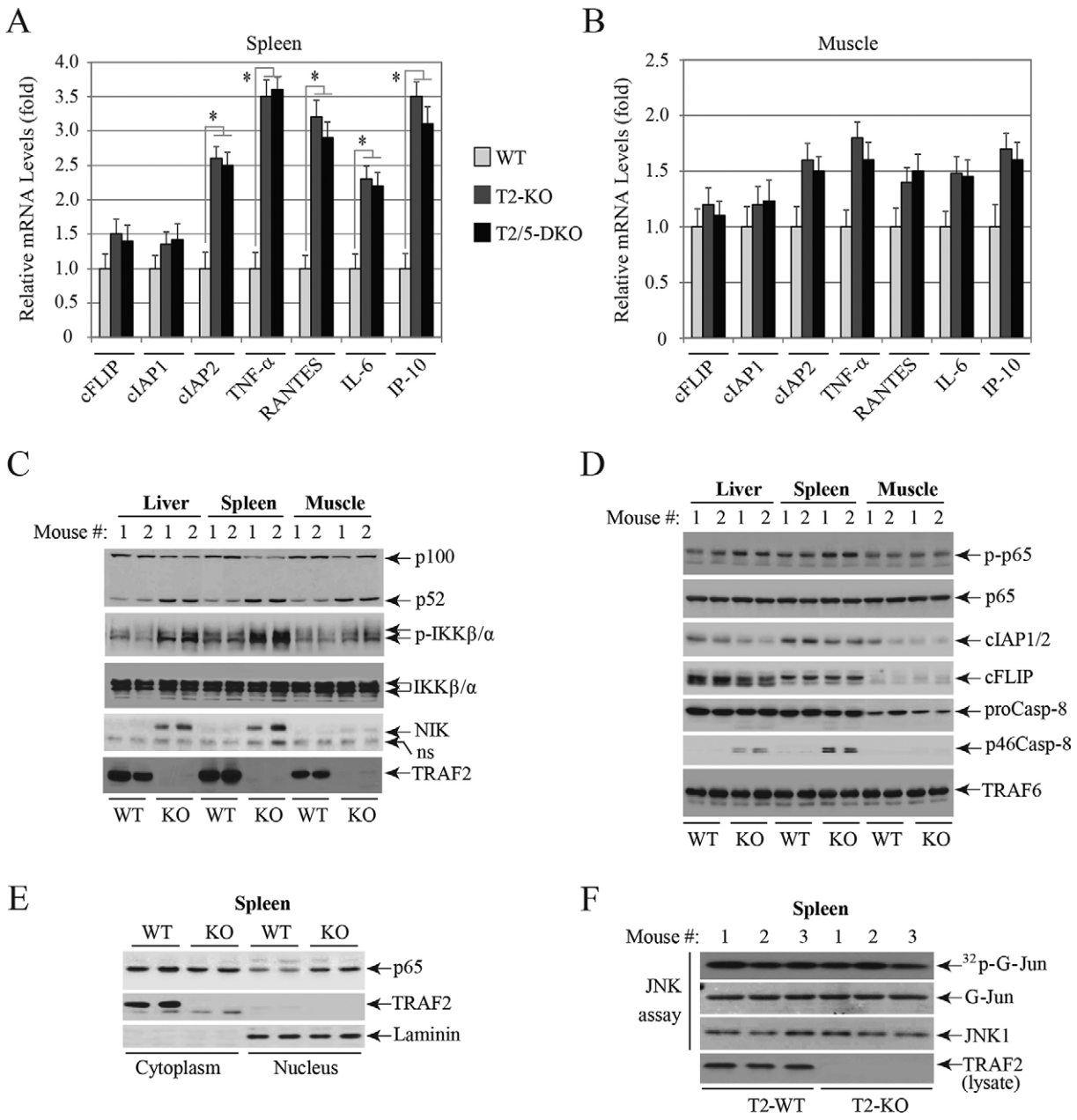

Fig. 2. TRAF2 suppresses NF-κB target gene expression and caspase-8 activity in spleen and liver. (A,B) Total RNA was extracted from the spleens and muscles of 7-day-old wild-type (WT), TRAF2-KO (T2-KO) and TRAF2/5-DKO (T2/5-DKO) mice (n=3 per genotype), and the expression of NF-κB target genes was determined by performing real-time RT-PCR. The relative expression level of each gene is presented as the ratio between the respective gene and the reference gene GAPDH, as mean±s.d. from three mice, *P<0.05 (Student's t-test). Levels are relative to WT. (C,D) Total protein samples were extracted from livers, spleens and muscles isolated from 7-day-old WT and TRAF2-KO (KO) mice, and p100 processing, IKK and p65 phosphorylation (p-IKKβ/α and p-p65), and FLIP (cFLIP), IAP1 and IAP2 (cIAP1/2), caspase-8 (proCasp-8) and NIK expression, as well as caspase-8 cleavage (p46Casp-8) were monitored by western blotting. ns, denotes a non-specific band. (E) Cytoplasmic and nuclear fractions were prepared from the spleens of 7-day-old WT and TRAF2-KO mice, and subcellular localization of NF-κB (p65) was determined by western blotting. (F) Total protein samples were extracted from the spleens of 7-day-old WT and TRAF2-KO mice, and then JNK was immunoprecipitated and subjected to *in vitro* kinase assays using GST–Jun (G-Jun) as substrate. ^{32}p-G-Jun, ^{32}P-labeled GST–Jun.

TNF/TRAF2-DKO and TNF/TRAF2/5-TKO mice succumb to a sublethal dose of TNFα

To assess the roles of TRAF2 and TRAF5 in TNFα-induced NF-κB target gene expression *in vivo*, we injected 4-month-old TNF-KO, TNF/TRAF2-DKO and TNF/TRAF2/5-TKO mice with TNFα, and attempted to examine gene expression 3 and 6 h later; however, approximately 100 min after injection, both TNF/TRAF2-DKO and TNF/TRAF2/5 TKO mice started to produce watery stool and to exhibit a shallow respiratory rate, and died ∼20 min after. In contrast, TNF-KO mice did not show any of these symptoms. The diarrhea and bradypnea observed in DKO and TKO mice prompted us to examine morphological changes and cell death in the lung and gastrointestinal tract of DKO mice. Notably, because we did not see any difference between DKO and TKO mice, we did not histologically analyze TKO mice. Consistent with previous reports (Lin et al., 2011), inflammatory cellular infiltrate and multifocal to diffuse crypt necrosis were observed throughout the small intestines of DKO mice (Fig. 6A) (data not shown). Approximately 2 h after

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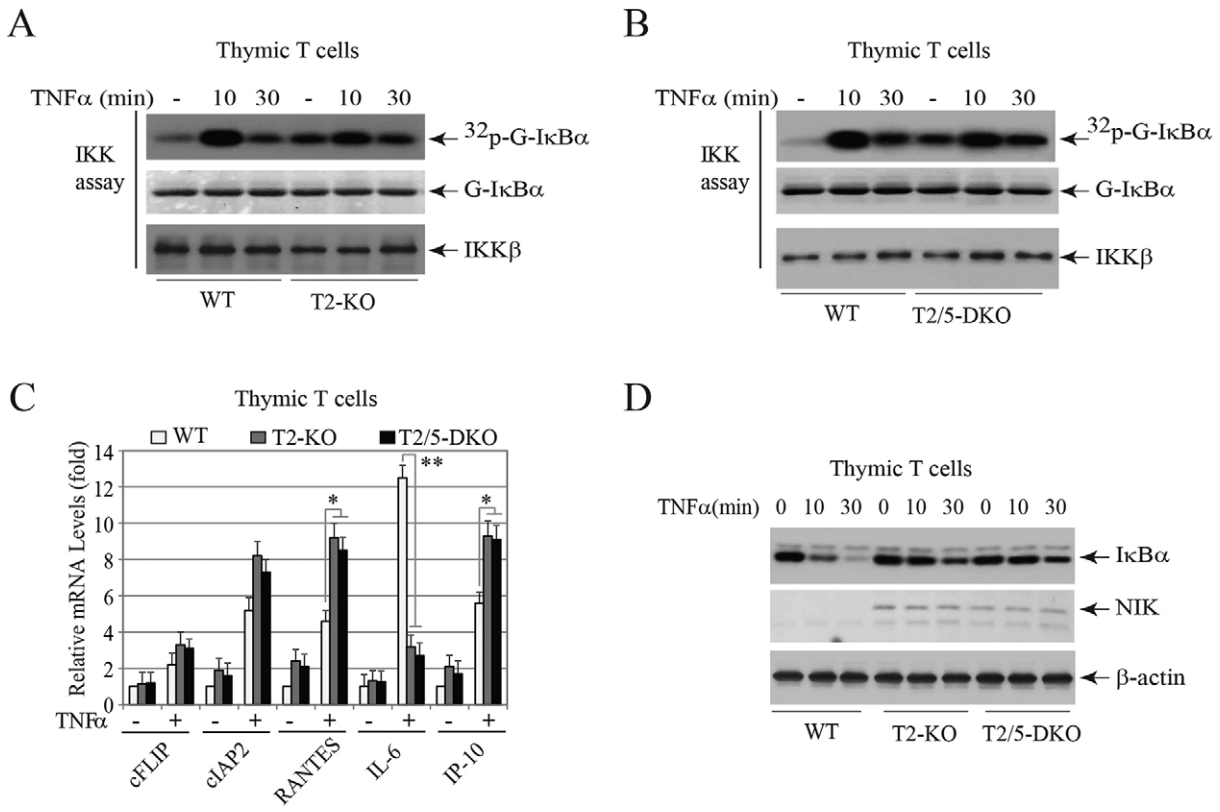

Fig. 3. TRAF2 suppresses the basal activity of the classical IKK complex in primary T cells. (A,B) Freshly isolated thymocytes were cultured in 5% FBS in RPMI for 4 h before being treated with mouse TNFα (5 ng/ml) as indicated. Classic IKK activity was then determined by kinase assays, as described in Fig. 1A. (C) Freshly isolated thymocytes were cultured in 5% FBS with RPMI for 4 h before TNFα (5 ng/ml) treatment for 2 h, and then the expression of NF-κB target genes was determined by performing real-time RT-PCR, as described in Fig. 2A. (D) Freshly isolated thymocytes were cultured in 5% FBS with RPMI for 4 h before TNFα (5 ng/ml) treatment as indicated, and then the degradation of IκBα and expression of NIK were monitored by western blotting. ^{32}p-G-IκBα, ^{32}P-labeled GST–IκBα; G-IκBα, GST–IκBα; TRAF2-KO, T2-KO; TRAF2/5-DKO, T2/5-DKO. *P<0.05, **P<0.01 (Student's t-test).

injection of TNFα, almost all enterocytes in the crypts underwent necrotic death, whereas villus enterocytes maintained relatively normal morphology. By contrast, multiple large vessels and airways in the lungs of DKO mice were cuffed by lymphocytes; however, we were unable to detect any morphological changes or increased apoptotic cells in the lungs of DKO mice following TNFα injection (Fig. 6B) (data not shown). Western blot analysis of lung and intestine lysates did not reveal increased cleavage of caspase-3 or FLIP in DKO mice after TNFα injection, though a small portion of caspase-8 had been processed to the moderately active p46casp-8 fragment (Fig. 6C). Comparatively, gene expression analysis revealed that TNFα injection caused increased expression of RANTES and inducible nitric oxide synthase (iNOS; also known as NOS2) in both DKO and TKO mice (Fig. 6D). These data suggest that TNFα, indeed, induces NF-κB target gene expression in DKO and TKO mice, and that the diarrhea and bradypnea observed in DKO and TKO mice following TNFα injection is most likely caused by massive necrosis in the crypts and the overexpression of NF-κB target genes in the lungs, but not by increased caspase-3 activation or apoptosis.

TRAF2-deficiency seems to cause increased basal IKK activation mainly in hematopoietic cells *in vivo*

Increased NF-κB target gene expression in the lungs of 4-month-old TNF/TRAF2- DKO mice following TNFα injection prompted us to examine basal IKK activity in the lungs and spleens. Interestingly, basal IKK activity was elevated in the lungs but not in the spleens of 4-month-old TNF/TRAF2-DKO mice compared to TNF-KO mice (Fig. 7A,B). Hematoxylin and eosin (H&E) staining and immunohistochemistry analyses revealed that the splenic white pulps in TNF/TRAF2-DKO mice were severely depleted of both T (marker CD3) and B (marker B220, also known as PTPRC) cells, whereas no clear morphological differences were observed in the muscles of TNF-KO and TNF/TRAF2-DKO mice (Fig. 7C). Given that the basal IKK activity was not elevated in the lungs of 7-day-old TRAF2-KO mice (Fig. 1D), the increased basal IKK activation in the lungs of 4-month-old TNF/TRAF2-DKO mice is most likely due to lymphocyte infiltration, which suggests that TRAF2 plays a more important role in regulation of basal IKK activity in hematopoietic cells *in vivo*.

TKO MEFs are more sensitive to TNFα-induced cell death than DKO counterparts

TRAF2/5-DKO mouse embryonic fibroblasts (MEFs) have been shown to be more sensitive to TNFα-induced cell death than TRAF2-KO counterparts (Tada et al., 2001). We found that TNF/TRAF2-DKO and TNF/TRAF2/5-TKO mice succumbed to a sublethal dose of TNFα at the same time (about 120 min after injection). It is possible that different types of cell exhibit differential sensitivities to TNFα and that autocrine TNFα production or immortalization could also alter TNFα sensitivity. To test these possibilities, we isolated primary thymic T cells and MEFs from the abovementioned DKO and TKO mice, and examined their sensitivity to TNFα. As shown in Fig. 7D,E,

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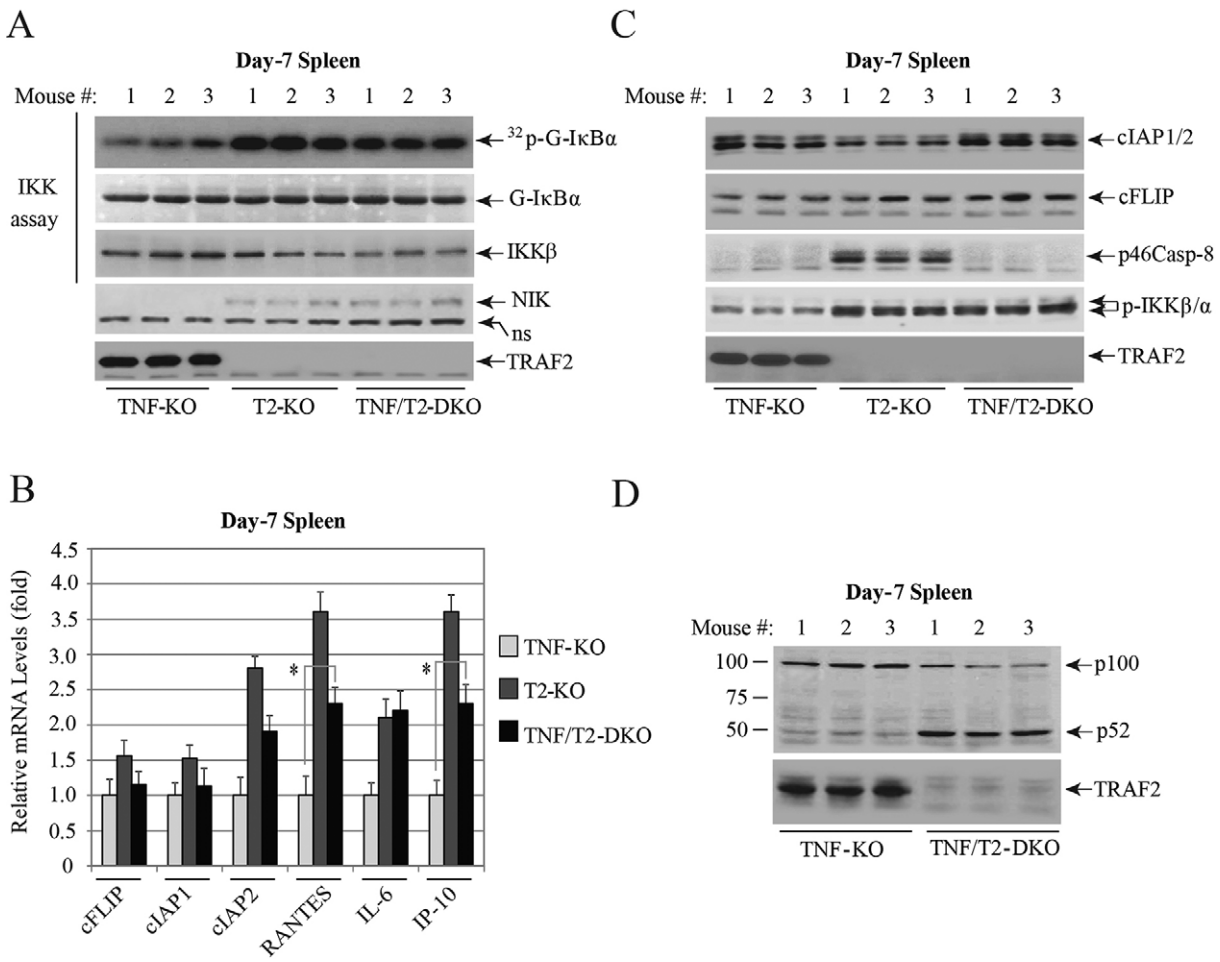

Fig. 4. Knockout of TNFα partially suppresses basal NF-κB activity in TRAF2-KO spleen. (A) Protein samples were extracted from the spleens of 7-day-old TNF-KO, TRAF2-KO (T2-KO) and TNF/TRAF2-DKO mice, and classic IKK activity was determined by kinase assays, as described in Fig. 1A, and expression of NIK was monitored by western blotting. (B) Total RNA was extracted from the spleens of 7-day-old mice (n=3 per genotype), and the expression of NF-κB target genes was determined by real-time RT-PCR, as described in Fig. 2A. (C,D) Expression and cleavage of IAP1 and IAP2 (cIAP1/2), FLIP (cFLIP) and caspase-8 (p46Casp-8), phosphorylation of IKK (pIKKβ/α), and processing of p100 to p52 in the spleens of 7-day-old mice were monitored by western blotting. ^{32}p-G-IκBα, ^{32}P-labeled GST–IκBα; G-IκBα, GST–IκBα; ns, denotes a non-specific band. *P<0.05 (Student's t-test).

primary TKO thymocytes were slightly more sensitive to TNFα-induced cell death than DKO counterparts, whereas TKO MEFs were clearly more sensitive to TNFα than DKO counterparts. These differences are not due to impaired NF-κB activation, because IKK kinase assays revealed that basal IKK activity was elevated and TNFα-induced delayed IKK activation was intact in TKO MEFs (Fig. 8A). In addition, stable expression of Flag–TRAF5 in TKO MEFs had no effect on NIK protein level or on basal and inducible phosphorylation of IκBα, but it clearly suppressed the susceptibility of TKO MEFs to TNFα-induced cell death (Fig. 8B,C). These data suggest that TRAF5 does contribute to the protection of cells from TNFα-induced cell death, independent of NF-κB activation.

Depletion of IAP1 and IAP2 also impairs TNFα-induced immediate, but not delayed, IKK activation

Depletion of IAP1 and IAP2 causes cell death in some cancer cells through NF-κB activation and autocrine TNFα production (Varfolomeev et al., 2007; Vince et al., 2007). To assess the role of IAP1 and IAP2 in TNFα-induced immediate IKK activation, we depleted IAP1 and IAP2 with Smac mimetics in primary TNF-KO MEFs. As shown in Fig. 8D, depletion of IAP1 and IAP2 resulted in elevated basal IKK activation and complete impairment of TNFα-induced immediate, but not delayed IKK, activation. These data further confirm that TRAF2, IAP1 and IAP2 cooperatively suppress basal IKK activity and play an essential role in TNFα-induced immediate IKK activation.

Knockdown of NIK suppresses basal IKK activity and NF-κB-dependent gene expression

NIK accumulation in TRAF2-KO and IAP1- and IAP2-depleted cells has also been shown to promote canonical IKK activation (Zarnegar et al., 2008). As expected, small interfering (si)RNA-mediated knockdown of NIK in TNF/TRAF2-DKO cells reduced the basal IKK activity, and suppressed both the basal and TNFα-induced expression of RANTES and IP-10, but it had no effect on the kinetics of TNFα-induced IKK activation (Fig. 8E,F). These data suggest that accumulation of NIK in TRAF2-KO cells promotes basal IKK activity but does not affect TNFα-induced IKK activation, and that elevated basal IKK activity in TRAF2-KO cells render the cells more responsive to TNFα-induced gene expression.

DISCUSSION

The TNFα–TNFR1 axis plays a crucial role in inflammatory responses and is one of the most studied signaling pathways;

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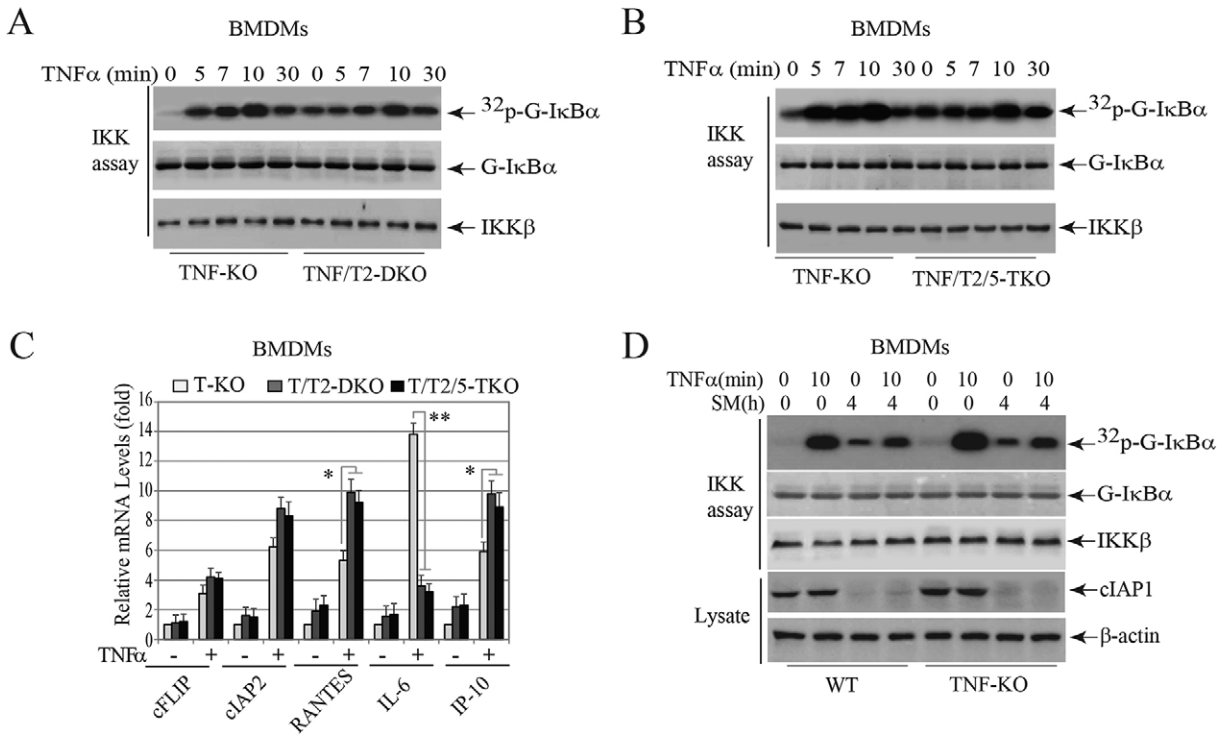

Fig. 5. TRAF2 is required for the immediate phase of IKK activation in BMDMs. (A,B) Bone marrow cells that had been isolated from 8-week-old mice were cultured in 15% FBS with DMEM containing 20% L929 conditioned medium for 10 days before TNFα (5 ng/ml) treatment, and then classic IKK activity was determined by using kinase assays. (C) BMDMs derived from the indicated mice were treated with TNFα (5 ng/ml) for 2 h, and the expression of NF-κB target genes was determined by performing real-time RT-PCR. Levels are relative to untreated TNF-KO (T-KO). (D) BMDMs were pre-treated with Smac mimetic (SM) for 4 h before being treated with TNFα (5 ng/ml) for 10 min, and then IKK activity was determined by performing kinase assays. ^{32}p-G-IκBα, ^{32}P-labeled GST–IκBα; cIAP1, IAP1; G-IκBα, GST–IκBα; T-KO, TNF-KO; T/T2-DKO, TNF/TRAF2-DKO; T/T2/5-TKO, TNF/TRAF2/5-TKO; WT, wild type. *P<0.05, **P<0.01 (Student's t-test).

however, our understanding of the molecular mechanisms underlying TNFR1-mediated NF-κB activation has been complicated by the accumulation of contradictory results (Alvarez et al., 2010; Habelhah et al., 2004; Mahoney et al., 2008; Varfolomeev et al., 2008; Wong et al., 2010; Yamamoto et al., 2006; Yin et al., 2009; Zhang et al., 2010, 2009). For example, it has been firmly believed that TNFα-induced NF-κB activation is partially impaired in TRAF2-KO cells and completely impaired in RIP1-KO cells. However, Nguyen et al. have reported previously that TRAF2-deficient macrophages overexpress certain NF-κB-regulated genes (e.g. TNFα and iNOS) following TNFα stimulation (Nguyen et al., 1999), and Wong et al. have reported recently that TNFα can normally activate NF-κB in RIP1-KO cells (Wong et al., 2010).

Through an extensive analysis of IKK activation and NF-κB target gene expression *in vitro* and *in vivo*, we discovered that: (i) TRAF2 suppresses the basal activity of the classic IKK complex in postnatal spleen and liver, as well as in primary hematopoietic cells and MEFs; (ii) TNFα-induced immediate IKK activation is completely impaired in TRAF2-KO, TRAF2/5-DKO and IAP1- and IAP2-depleted primary cells; (iii) TNFα can still activate the delayed phase of IKK in the absence of TRAF2 and TRAF5 or IAP1 and IAP2 expression, albeit weakly; (iv) TNFα stimulation results in increased expression of a subset of NF-κB target genes in both TRAF2-KO and TRAF2/5-DKO cells; (v) a sublethal dose of TNFα kills TNF/TRAF2-DKO and TNF/TRAF2/5-TKO mice by inducing necrosis in small intestine crypts and hyperactivation of NF-κB target gene expression in the lungs in a short time period that is not long enough for full activation of caspase-3 and the apoptotic cascades; and (vi) T and B cells migrate from the spleen to the lung and gastrointestinal tract in adult TNF/TRAF2-DKO mice. These *in vitro* and *in vivo* data provide compelling evidence that TRAF2, but not TRAF5, plays a non-redundant dual role in regulating basal and TNFα-induced activation of the canonical NF-κB pathway, and thus clarifies conflicting observations regarding the roles of these proteins in TNFR1 signaling (see below).

In the case of TNFR1 signaling, TRAF2, IAP1 and IAP2 cooperatively activate canonical NF-κB by catalyzing the non-canonical ubiquitylation of RIP1 and themselves (Bradley and Pober, 2001; Hayden and Ghosh, 2008). Notably, recombinant IAP1 and IAP2 purified from bacteria exhibit strong E3 ligase activity and are able to conjugate nearly all types of ubiquitin linkages in *in vitro* ubiquitylation assays; however, the E3 ligase activity of TRAF2 remains controversial (Workman and Habelhah, 2013). A recent structural study has revealed that except for TRAF6, all other TRAF proteins expressed and purified from bacteria do not exhibit E3 ligase activity (Yin et al., 2009). In the case of TRAF2, nine amino acids between the RING domain and first zinc finger motif sterically interfere with the interaction between the RING domain and E2 enzymes (Yin et al., 2009). Nevertheless, in TRAF2-KO, TRAF2/5-DKO and IAP1- and IAP2-depleted cells, TNFα-induced RIP1 ubiquitylation is impaired and IκBα degradation is incomplete (Feltham et al., 2010; Lee et al., 2004; Mahoney et al., 2008). We found in this study that the immediate, but not the delayed, phase of IKK activation is completely impaired in TRAF2-KO, TRAF2/5-DKO and IAP1- and IAP2-depleted primary cells. These results are identical to those we have reported recently in RIP1-KO MEFs (Blackwell et al., 2013). Collectively, those published findings and our current data suggest that TRAF2,

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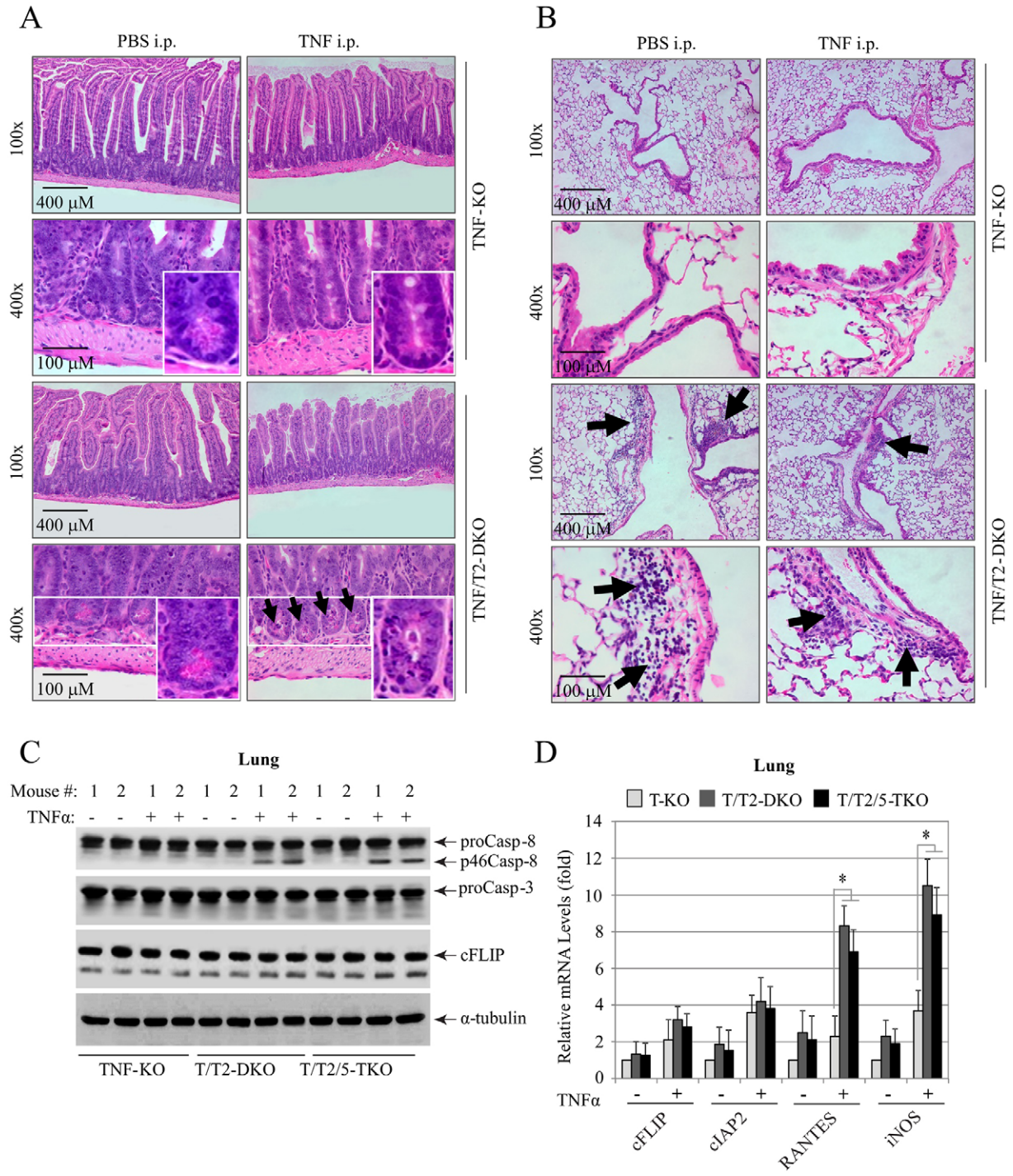

Fig. 6. TNFα injection induces overexpression of a subset of NF-κB target genes in TNF/TRAF2-DKO and TNF/TRAF2/5-TKO mice. (A,B) 4-month-old TNF-KO and TNF/TRAF2-DKO mice were injected intraperitoneally (i.p.) with recombinant mouse TNFα (0.25 mg/kg), and immediately after mice died, the gut roll (A) and lung (B) were fixed, and sections were stained with H&E to show gross structure. Arrows show the crypts (A) and the lymphocytes (B). Insets in A show the magnified view of the enterocytes in the crypts. The magnifications at which samples were viewed are indicated. (C,D) TNF(T)-KO, TNF/TRAF2 (T/T2)-DKO and TNF/TRAF2/5 (T/T2/5)-TKO mice were injected intraperitoneally with mouse TNFα (0.25 mg/kg), and protein and RNA samples were extracted from the lungs immediately after death. Then, the expression and cleavage of caspase-3 (proCasp-3), caspase-8 (proCasp-8 to p46Casp-8) and FLIP (cFLIP) were monitored by western blotting, and the expression of NF-κB target genes was determined by real-time RT-PCR. Levels are relative to untreated TNF-KO (T-KO). Data are mean±s.d. *P<0.05 (Student's t-test).

IAP1 and IAP2 cooperatively catalyze RIP1 ubiquitylation to trigger immediate IKK activation and that TRAF5 has no substantial role in this process. In fact, TRAF2 interacts with IAP1, IAP2 and TRADD, whereas TRAF5 does not interact with any of these proteins (Hayden and Ghosh, 2008; Varfolomeev et al., 2007; Vince et al., 2007; Wang et al., 2008).

Analysis of IκBα phosphorylation and degradation is the most commonly used method to assess stimulus-induced NF-κB

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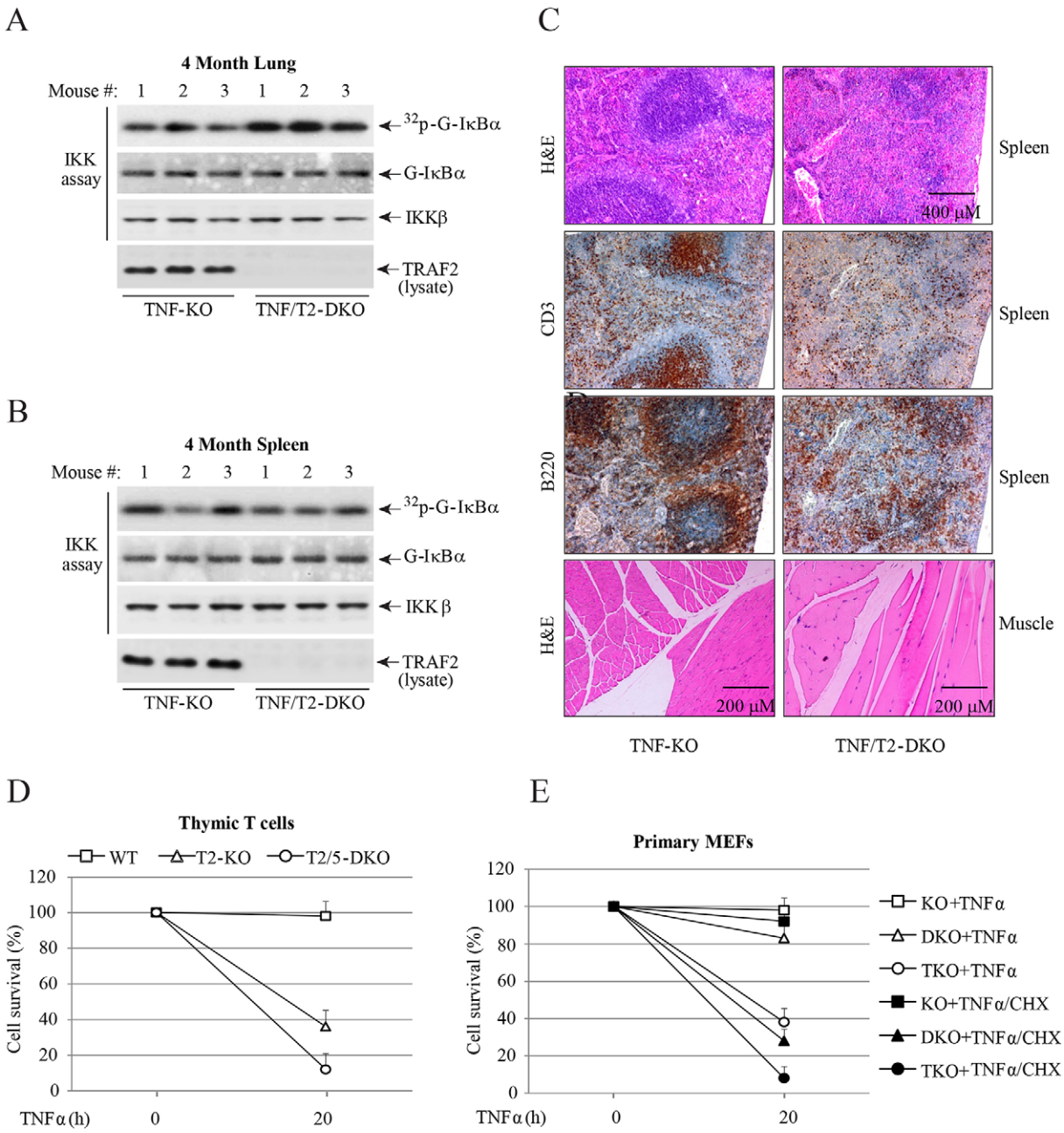

Fig. 7. TRAF2 is essential for the homeostasis of T and B cells in spleen. (A,B) Lungs and spleens were removed from 4-month-old TNF-KO and TNF/TRAF2 (TNF/T2)-DKO mice, and IKK activity was determined by performing kinase assays. (C) Spleens and muscles were removed from 4-month-old TNF-KO and TNF/TRAF2-DKO mice, and fixed, and then sections were stained with H&E to show gross structure, and for CD3 and B220 to detect splenic T and B cells, respectively. (D,E) Primary thymic T cells and MEFs were treated with mouse TNFα (5 ng/ml) or mouse TNFα (5 ng/ml) plus CHX (0.2 μg/ml) (TNFα/CHX), and the rate of cell death was assessed by using the Trypan Blue exclusion assay 20 h later. Data are mean±s.d. ^{32}p-G-IκBα, ^{32}P-labeled GST–IκBα; G-IκBα, GST–IκBα; KO, TNF-KO; DKO, TNF/TRAF2-DKO; TKO, TNF/TRAF2/5-TKO mice.

activation (Workman and Habelhah, 2013). Previous conclusions that knockout of both TRAF2 and TRAF5 or depletion of IAP1 and IAP2 abolishes TNFα-induced NF-κB activation are based on analyses of IκBα degradation (Tada et al., 2001; Workman and Habelhah, 2013). We found that the classic IKK complex is constitutively activated to a certain degree in TRAF2/5-DKO and IAP1- and IAP2-depleted cells, and that TNFα-induced delayed IKK activation is not completely impaired in these cells. This suggests that impaired IκBα degradation in TRAF2/5-DKO cells is not due to impaired IKK activation, but rather due to constitutive phosphorylation, degradation and re-synthesis of IκBα in these cells, which partially mask the complete TNFα-induced degradation of IκBα. In line with this, inhibition of proteasome activity resulted in IκBα accumulation in both TRAF2-KO and TRAF2/5-DKO cells.

Analysis of NF-κB target gene expression is another common method used to assess stimulus-induced NF-κB activation. An earlier study has shown that TRAF2-KO macrophages overproduce TNFα and NO in response to TNFα stimulation (Nguyen et al., 1999). A more recent study shows that TRAF2-KO T cells produce

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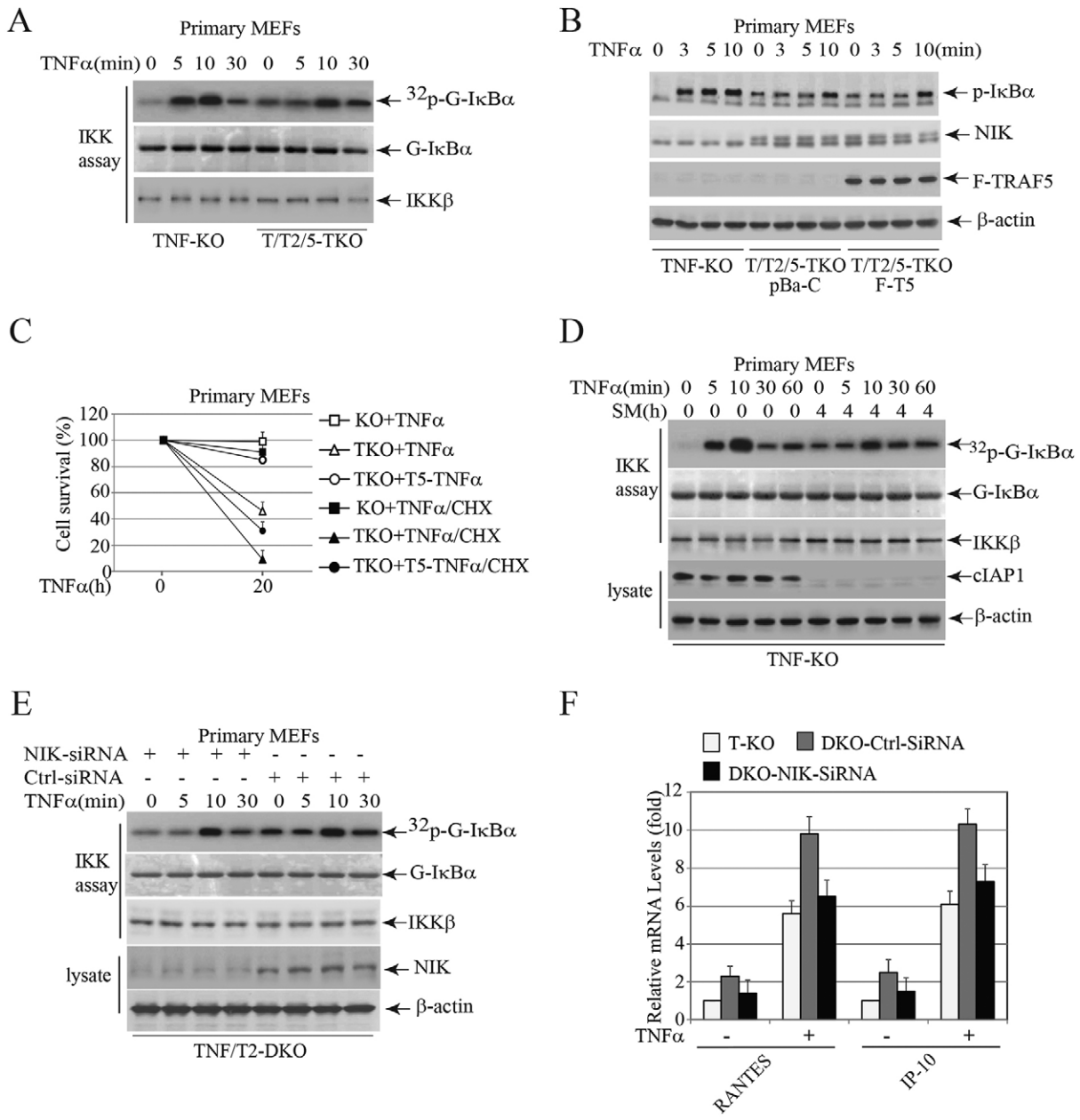

Fig. 8. TRAF5 regulates TNFα-induced cell death but not NF-κB activation. (A) Primary MEFs from TNF-KO and TNF/TRAF2/5-TKO embryos were treated with TNFα (5 ng/ml), and IKK activity was then determined by performing kinase assays. (B) Primary TNF/TRAF2/5-TKO MEFs were stably transduced with control pBabe (pBa-C) or Flag–TRAF5 (F-TRAF5), treated with TNFα (5 ng/ml) as indicated, and then the phosphorylation of IκBα and expression of NIK were monitored by western blotting. (C) Primary TNF-KO (KO) and TNF/TRAF2/5-TKO (TKO) MEFs stably transduced with pBa-C or Flag– TRAF5 (T5) were treated with mouse TNFα (5 ng/ml) or mouse TNFα (5 ng/ml) plus CHX (0.2 µg/ml) (TNFα/CHX), and the rate of cell death was assessed by using the Trypan Blue exclusion assay 20 h later. KO, TNK-KO. (D) Primary MEFs were treated with Smac mimic (SM) (200 ng/ml) for 4 h prior to stimulation with TNFα, and IKK activity was then determined by performing kinase assays. (E,F) Primary MEFs were transfected with an siRNA specific to mouse NIK (NIK-siRNA) or a control siRNA (Ctrl-siRNA), and 72 h after transfection, the MEFs were stimulated with TNFα (5 ng/ml), and IKK activation (IKKβ) and RANTES and IP-10 expression were determined by performing kinase assays and real-time RT-PCR. Levels are relative to untreated TNF-KO (T-KO). Data are mean±s.d. ^{32}p-G-IκBα, ^{32}P-labeled GST–IκBα; DKO, TNF/TRAF2-DKO; G-IκBα, GST–IκBα; p-IκBα, phosphorylated IκBα; T-KO, TNF-KO;

elevated levels of Th1 and Th17 cytokines independent of TNFα signaling (Lin et al., 2011). In addition, depletion of IAP1 and IAP2 with small-molecule IAP antagonists elicits NF-κB activation and TNFα production, resulting in TNFα-dependent apoptosis in a subset of cancer cells (Varfolomeev et al., 2007; Vince et al., 2007). Our analysis of NF-κB-dependent gene expression revealed that although IAP1 and FLIP are expressed at normal levels, the basal expression of RANTES and IP-10 is significantly increased in the spleen and primary T cells derived from TRAF2-KO and TRAF2/5-DKO mice independent of TNFα signaling. Moreover, although TNFα stimulation significantly increased the expression of RANTES and IP-10 in TRAF2-KO and TRAF2/5-DKO cells compared to that in wild-type cells, IL-6 expression was almost completely impaired. Notably, the expression of a subset of NF-κB target genes, such as IP-10 and RANTES, is regulated by both the canonical and non-canonical NF-κB pathways (Hayden and Ghosh,

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2008; Hoffmann et al., 2003, 2002; Wang et al., 1998). Comparatively, efficient IL-6 expression requires both NF-κB and Jun activity, because TNFα-induced IL-6 expression is impaired in both JNK1- and JNK2-DKO and p65-KO MEFs (Okazaki et al., 2003; Ventura et al., 2003). Altogether, these published findings and our data suggest that the elevated expression of a subset of NF-κB target genes in TRAF2-KO and TRAF2/5-DKO cells following TNFα stimulation is due to two events: (i) constitutive activation of both the canonical and noncanonical NF-κB pathways to a certain degree in these cells before stimulation and (ii) inducible activation of the delayed phase of IKK after stimulation. Of note, the canonical NF-κB pathway is only partially activated in TRAF2-KO and TRAF2/5-DKO cells, whereas the noncanonical NF-κB pathway is fully activated. The canonical NF-κB pathway is subject to strong negative-feedback regulation, involving the recruitment of inhibitory proteins (e.g. TRAF1 and A20), dephosphorylation of IKK at its T-loop, cleavage and degradation of p65, and the rapid re-synthesis of IκBα (Bonizzi and Karin, 2004; Hayden and Ghosh, 2008). Therefore, it is possible that these negative regulators also exhibit elevated basal activities in TRAF2-KO and TRAF2/5-DKO cells.

In TRAF2-KO and TRAF2/5-DKO cells, TNFα triggers caspase-8-mediated cleavage and subsequent degradation of FLIP within 2–3 h after stimulation, both of which are early and crucial events that culminate in necrotic and apoptotic cell death (Budd et al., 2006; Nakajima et al., 2006). Consistent with previous reports (Tada et al., 2001; Zhang et al., 2009), we also observed that primary TNF/TRAF2/5-TKO cells are more susceptible to TNFα-induced cell death than TNF/TRAF2-DKO counterparts; however, TNFα-induced NF-κB activation and target gene expression were comparable between the TKO and DKO cells. Although TRAF5 neither has E3 ligase activity nor interacts with TRADD or IAP1 and IAP2, it has been shown to interact with RIP1 following TNFα stimulation (Tada et al., 2001). Thus, it is possible that TRAF5 interacts with RIP1 to suppress its pro-apoptotic activity and thereby indirectly affects NF-κB activation in response to treatment with TNFα. In the case of treatment with TNFα *in vivo*, both TNF/TRAF2-DKO and TNF/TRAF2/5-TKO mice succumbed to death within about 2 h of TNFα injection owing to diarrhea and labored breathing. Diarrhea is most likely caused by massive necrosis in the small intestine crypts, suggesting that cryptic enterocytes are exceptionally susceptible to TNFα in the absence of TRAF2. In contrast, histological and western blot analyses did not reveal any increase in apoptotic cells or morphological changes in the lung of TNF/TRAF2-DKO mice. However, RANTES and iNOS were significantly upregulated in the lung of both TNF/TRAF2-DKO and TNF/TRAF2/5-TKO mice within 2 h of TNFα injection, suggesting that respiratory distress in DKO and TKO mice following TNFα injection is caused by increased expression of certain NF-κB target genes. These *in vivo* data confirm our *in vitro* findings that TNFα can activate the canonical NF-κB pathway in the absence of both TRAF2 and TRAF5.

MATERIALS AND METHODS

Antibodies and reagents

Antibodies and reagents were obtained as follows: anti-TRAF2 (C-20; #sc-876; 1:1000 dilution) and anti-IKKγ (FL-419; #sc-8330; 1:1000 dilution) antibodies and siRNAs (#sc-36066) for mouse NIK and control siRNA-A (#sc-37007) were from Santa Cruz Biotechnology; anti-FLIP (NF6; #ALX-804-428-C050; 1:500 dilution) antibody from Alexis (San Diego, CA); an antibody recognizing both IAP1 and IAP2 (MAB3400; 1:500 dilution) was from R&D Systems; and anti-phosphorylated-IκBα (14D4; #2859; 1:1000 dilution), anti-IκBα (#9242; 1:1000 dilution), anti-IKKβ (#2684; 1:500 dilution), anti-caspase-3 (#9662; 1:1000 dilution), anti-mouse caspase-8 (#4927; 1:500), anti-mouse cleaved caspase-8 (D5B2; #8592; 1:1000 dilution), anti-phosphorylated IKK (#2697; 1:500 dilution; recognizes both IKKa and IKKb phosphorylation) and anti-NIK (#4994; 1:300 dilution) antibodies were purchased from Cell Signaling. Recombinant mouse TNFα was from Roche; anti-IgG secondary antibodies and protease and phosphatase inhibitor cocktails were from Pierce.

Generation of DKO and TKO mice

Traf2$^{+/-}$ (C57BL/6) and *Traf5*$^{-/-}$ (C57BL/6) mice have been described previously (Kraus et al., 2008; Yeh et al., 1997), and *Tnf*$^{-/-}$ (C57BL/6) mice were purchased from The Jackson Laboratory (Bar Harbor, ME). The frequency of viable TRAF2-KO mice increases with a mixed genetic background of C57BL/6 and BALB/c (Yeh et al., 1997); therefore, we first crossed *Traf2*$^{+/-}$ mice with BALB/c mice to generate the *Traf2*$^{+/-}$ F1 hybrid mice, which were then intercrossed or crossed with *Traf5*$^{-/-}$ or *Tnf*$^{-/-}$ mice to generate TRAF2-KO, TRAF2/5-DKO, TNF/TRAF2-DKO and TNF/TRAF2/5-TKO mice as previously described (Nguyen et al., 1999; Tada et al., 2001). Mice were housed in the University of Iowa Mouse Facility under specific pathogen-free conditions. All mouse procedures were approved by the University of Iowa Institutional Animal Care and Use Committee.

Isolation of primary cells

For MEFs, embryos at 12.5–13.5 days of gestation were dissected from the uterus, and the head and liver were removed. Fetal tissue was then minced, digested with trypsin, washed with PBS and plated into two 100-mm dishes. All MEFs were used for experiments before passage eight (within three weeks). For isolation of BMDMs, mouse femurs were removed of muscles and crushed by mortar and pestle in 12 ml of Dulbecco's modified Eagle's medium (DMEM), and then the bone marrow materials were pipetted up and down to bring the cells into a single-cell suspension. The single-cell suspensions were filtered through a 70-μM nylon strainer and cultured in DMEM containing 15% fetal bovine serum (FBS) and 20% L929 conditioned medium for 10 days with medium changes every other day. For isolation of thymic T cells, thymic lobes were crushed in 2 ml of medium to disperse thymocytes, which were filtered through a 70-μM nylon strainer, and cultured in 5% FBS with RPMI for 4 h before stimulation with TNFα.

IKK immunokinase assay

To extract protein samples from mouse tissues (spleen, liver, lung and muscle), the tissues were excised immediately after euthanasia, diced, washed twice with ice-cold PBS and then homogenized in lysis buffer (20 mM HEPES pH 7.4, 0.5% Triton X-100, 350 mM NaCl, 1 mM EDTA, 20% glycerol, 1 mM DTT, 0.2 mM PMSF, 1× cocktail inhibitors of protease and phosphatases). After 30 min of incubation on ice with gentle agitation, lysates were cleared by centrifugation at 13,500 ***g*** for 15 min at 4°C, snap frozen in liquid nitrogen and stored at −80°C until analysis. For primary cells, the cells were washed once with ice-cold PBS, and protein samples were extracted with the same lysis buffer for 30 min on ice. The classic IKK complex was immunoprecipitated from the extracts with anti-IKKγ antibody, washed extensively, and then subjected to *in vitro* kinase assays as described previously (Habelhah et al., 2004).

Western blot analysis and real-time RT-PCR

Western blot analysis and real-time RT-PCR were performed as described previously (Blackwell et al., 2013).

Histology

Mouse spleen, lung and muscle were fixed in 10% neutral buffered formalin for 2–3 days, embedded in paraffin, sectioned with a microtome set at 5 μm and stained with H&E according to standard procedures (Hochstedler et al., 2013). For immunohistochemical analysis of splenic T and B cells, spleen sections were stained for CD3 and B220 to detect T and B cells, respectively. Gut rolls were prepared without removing the stool and fixed in the same 10% neutral buffered formalin for 2–3 days prior to H&E staining.

Preparation of retroviral supernatants and infection of TNF/TRAF2/5-TKO MEFs

HEK293T cells at 60–70% confluence were co-transfected with 2 µg of pMD.OGP (encoding gag-pol), 2 µg of pMD.G (encoding vesicular stomatitis virus G protein) and 2 µg of pBabe-Flag-TRAF5 or an empty vector (pBabe-puro) by using the standard calcium phosphate precipitation method. At 48 h after transfection, the viral supernatant was collected and then immediately used for the infection of the MEFs in the presence of 4 µg/ml polybrene for 6 h. At 48 h after infection, cells were selected with puromycin (2.0 µg/ml) for 3 days, and resistant cells were pooled and used for the functional experiments.

Statistical analysis

Data are expressed as the mean±s.d. and, for real-time RT-PCR analyses, the relative induction of NF-κB target genes was normalized to the expression level of reference gene GAPDH, and compared with the wild-type or untreated controls (set to 1). Data are representative of at least three experiments. Statistical analysis was performed with the paired Student's *t*-test, and $P<0.05$ was considered statistically significant.

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Competing interests

The authors declare no competing or financial interests.

Author contributions

H.H. had full access to all of the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis. L.Z. and H.H. designed the study. L.Z., K.B., L.M.W., K.N.G.-C. and A.K.O. performed the experiments and analyzed the data. L.Z., K.N.G.-C., G.A.B. and H.H. discussed the data and wrote the manuscript

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