LETTERS

Critical role of TRAF3 in the Toll-like receptor-dependent and -independent antiviral response

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Type I interferon (IFN) production is a critical component of the innate defence against viral infections[1]. Viral products induce strong type I IFN responses through the activation of Toll-like receptors (TLRs) and intracellular cytoplasmic receptors such as protein kinase R (PKR)[2–12]. Here we demonstrate that cells lacking TRAF3, a member of the TNF receptor-associated factor family, are defective in type I IFN responses activated by several different TLRs. Furthermore, we show that TRAF3 associates with the TLR adaptors TRIF and IRAK1, as well as downstream IRF3/7 kinases TBK1 and IKK-ε, suggesting that TRAF3 serves as a critical link between TLR adaptors and downstream regulatory kinases important for IRF activation. In addition to TLR stimulation, we also show that TRAF3-deficient fibroblasts are defective in their type I IFN response to direct infection with vesicular stomatitis virus, indicating that TRAF3 is also an important component of TLR-independent viral recognition pathways. Our data demonstrate that TRAF3 is a major regulator of type I IFN production and the innate antiviral response.

TNF receptor-associated factor (TRAF) family members, comprising TRAF1–6, are proposed to be adaptor molecules linking upstream receptor signals to downstream gene activation. Although multiple TRAFs are required for NF-κB and JNK activation by the TNF receptor members, including CD40 and TNFR2, TRAF6 was thought to be the sole TRAF family adaptor involved in interleukin (IL)-1 receptor and TLR signalling[13,14]. The function of TRAF3, a particularly elusive member of the TRAF family, has not yet been clearly defined[15]. TRAF3 was initially used as bait in a yeast two-hybrid screen several years ago to identify TRAF family member-associated NF-κB activator (TANK), which is an adaptor that was later found to associate with the IRF3 and IRF7 kinases TBK1 and IKK-ε[16–19]. NAK-associated protein 1 (NAP1), a TANK homologue, has recently been suggested to be involved in IRF3 activation[20]. Because the activation of IRF3/7 is an important event for type I IFN induction, this raises the possibility that TRAF3 could be directly involved in the IFN pathway[11,21,22]. Because TRAF3 deficiency results in early postnatal lethality, we generated bone marrow-derived macrophages (BMMs) from irradiated C57BL/6 mice, reconstituted with *Traf3*$^{+/+}$ and *Traf3*$^{-/-}$ fetal liver cells[15]. To study the involvement of TRAF3 in TLR signalling, *Traf3*$^{+/+}$ and *Traf3*$^{-/-}$ BMMs were stimulated with poly(I:C), a synthetic double-stranded RNA, and lipopolysaccharide (LPS), the ligands for TLR3 and TLR4, respectively. Notably, the induction of *Ifnb* (which encodes IFN-β) and subsequent IFN target genes in response to poly(I:C) and LPS was markedly reduced in TRAF3-deficient BMMs (Fig. 1a and Supplementary Fig. 3a), whereas no defect was observed in the activation of NF-κB or the induction of inflammatory-related genes such as *IκBα* and *KC* in *Traf3*-deficient BMMs (Fig. 1a and Supplementary Fig. 2d). *Traf3*$^{-/-}$ BMMs were not inherently defective in their ability to produce type I IFNs, as infection with *Listeria monocytogenes* resulted in potent IFN-β induction in these cells (Supplementary Fig. 4). Furthermore, conditioned media from poly(I:C)-treated wild-type BMMs potently blocks the replication of both murine gammaherpesvirus 68 (MHV68) and vesicular stomatitis virus (VSV) in NIH3T3 cells (Fig. 1b and Supplementary Fig. 3)[11]. In contrast, conditioned media from poly(I:C)-treated *Traf3*$^{-/-}$ BMM cultures had no effect on viral replication, demonstrating that TRAF3 is critical for the ability of TLR-stimulated cells to inhibit viral replication.

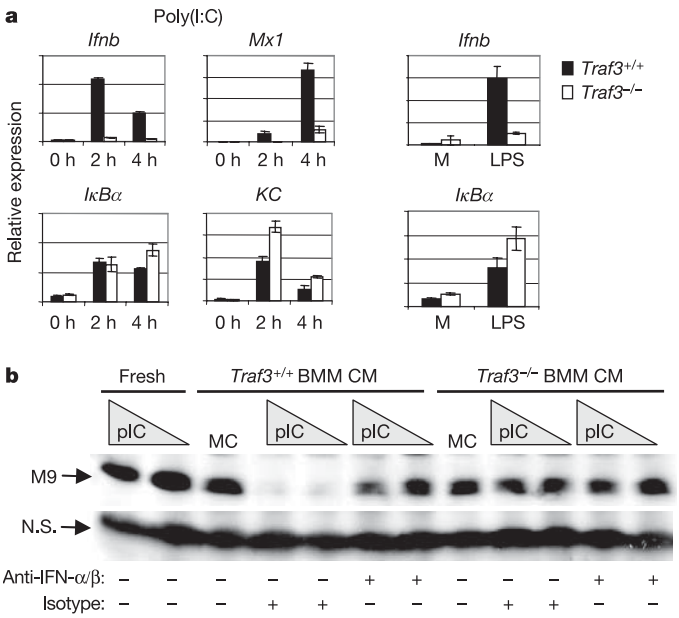

Figure 1 | Induction of the type I IFN response by TLR3 and TLR4 requires TRAF3. a, RNA from *Traf3*$^{+/+}$ or *Traf3*$^{-/-}$ BMMs stimulated with poly(I:C) (1 μg ml^{-1}) or LPS (20 ng ml^{-1}) was assayed for the expression of the indicated mRNA transcripts by Q-PCR. Error bars indicate ±s.d. between duplicates. M, media control. **b**, NIH3T3 cells were pre-treated for 3 h with conditioned media from BMMs stimulated with poly(I:C) (0.1 or 1 μg ml^{-1}) in the presence of anti-IFN-α/β or nonspecific rabbit IgG (20 μg ml^{-1}). Cells were then infected with MHV68. Twenty-four hours after infection, MHV68 replication was assayed by immunoblotting for MHV68 M9 protein. MC, media control; N.S., non-specific band.

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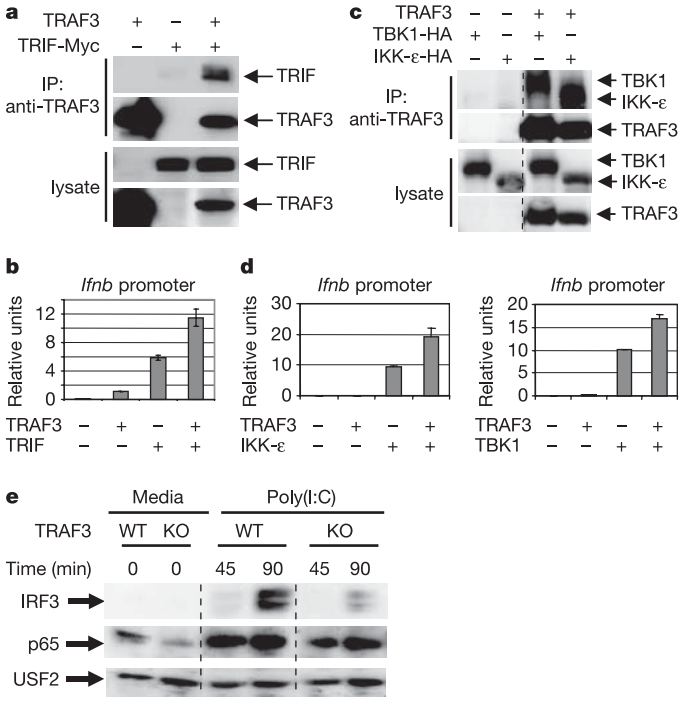

Figure 2 | TRAF3 associates with IFN pathway mediators and is required for TLR3-induced IRF3 activation. **a**, TRAF3 associates with Myc-TRIF when co-expressed in 293T cells by immunoprecipitation assay. **b**, TRAF3 synergizes with TRIF when co-expressed in 293T cells to activate *Ifnb* promoter by luciferase assay. **c**, TRAF3 associates with both haemagglutinin (HA)-TBK1 and HA-IKK-ε when co-expressed in 293T cells by immunoprecipitation assay. **d**, TRAF3 synergizes with both HA-TBK1 and HA-IKKε when co-expressed in 293T cells to activate *Ifnb* promoter by luciferase assay. **e**, Nuclear extracts from poly(I:C)-stimulated BMMs were monitored for the appearance of IRF3 and the p65 subunit of NF-κB by immunoblotting. KO, knockout; WT, wild type. Error bars in **b** and **d** indicate ±s.d. between duplicates.

TRIF is a critical adaptor molecule required for IFN production after poly(I:C) and LPS stimulation[23,24]. Our finding that *Traf3*$^{-/-}$ cells were defective in poly(I:C)- and LPS-mediated *Ifnb* upregulation suggested that TRAF3 might serve as an adaptor molecule linking TRIF to downstream IRF3 activation. In support of this hypothesis, we found that TRAF3 both associated with TRIF and significantly enhanced TRIF-mediated *Ifnb* promoter activity (Fig. 2a, b). Similarly, TRAF3 also associated with both TBK1 and IKK-ϵ, and synergized with both IRF3 kinases to enhance their activation of the *Ifnb* promoter (Fig. 2c, d). To determine whether TRAF3 is specifically involved in the activation and nuclear translocation of IRF3, nuclear extracts from poly(I:C)-treated wild-type and *Traf3*$^{-/-}$ BMMs were analysed for the presence of IRF3. Strong IRF3 nuclear translocation was detected in poly(I:C)-stimulated wild-type BMMs compared to only trace amounts of nuclear IRF3 in TRAF3-deficient cells (Fig. 2e). Together, these observations demonstrate that TRAF3 is a critical adaptor molecule for TRIF-mediated IRF3 activation and IFN-β production.

Plasmacytoid dendritic cells (pDCs) are thought to be the major producers of type I IFNs *in vivo*[25]. TLR9 and TLR7 activation induces potent MyD88- and IRAK1-dependent type I IFN production in pDCs[4–10,26]. To address whether TRAF3 is also involved in TLR7/9-dependent type I IFN induction, Flt3-ligand-derived dendritic cells were generated from *Traf3*$^{+/+}$ and *Traf3*$^{-/-}$ bone marrow and stimulated with CpG and R848, synthetic ligands for TLR9 and TLR7, respectively. IFN-α production was severely defective in the TRAF3-deficient cells treated with either TLR9 or TLR7 agonist (Fig. 3a and Supplementary Fig. 5). These findings were further confirmed in purified pDCs (Fig. 3b, c and Supplementary Figs 6 and 7). Bone marrow cells for these experiments were isolated from *Traf3*$^{+/+}$*p100*$^{-/-}$ and *Traf3*$^{-/-}$*p100*$^{-/-}$ mice, which survive into adulthood (J. He *et al.*, manuscript in preparation).

It has recently been demonstrated that IRAK1 is specifically required for TLR9-mediated IFN-α production, whereas it is dispensable for TLR9-induced inflammatory responses[26]. Our results reveal a similar phenotype in TRAF3-deficient cells. In fact, TRAF3 associated with IRAK1 when co-expressed in 293T cells and strongly synergized with IRAK1 and IRF7 to activate *Ifna4* promoter in a reporter assay (Fig. 3d, e). Thus, TRAF3 might function in a complex with IRAK1 to phosphorylate directly IRF7, or facilitate the

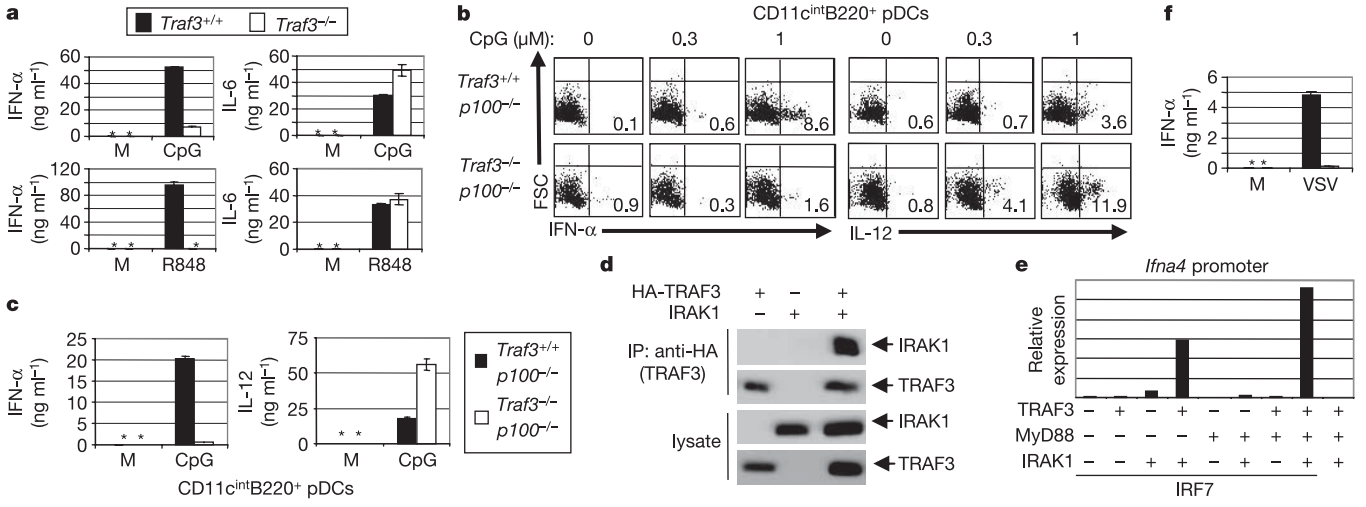

Figure 3 | TRAF3 is required for TLR7/9-dependent type I IFN production. **a**, **f**, Twenty-four-hour supernatants from Flt3-ligand-derived dendritic cells, stimulated with CpG D19 (1 μM), R848 (200 nM) (**a**) or infected with VSV (multiplicity of infection (MOI) of 1) (**f**), were analysed for the levels of IFN-α or IL-6 by ELISA (asterisk indicates not detected). M, media control. **b**, *p100*$^{-/-}$*TRAF3*$^{+/+}$ and *p100*$^{-/-}$*TRAF3*$^{-/-}$ Flt3-ligand-derived dendritic cells were stimulated with CpG D19 for 8 h, and cells were analysed for the presence of intracellular IFN-α and IL-12. **c**, CD11c^{int}B220^{+} pDCs were purified by cell sorting from Flt3-ligand-derived dendritic cells and stimulated with CpG D19 for 24 h. IL-12 and IFN-α levels were analysed by ELISA. **d**, **e**, TRAF3 associates and synergizes with IRAK1 when co-expressed in 293T cells. Error bars in **a**, **c** and **f** indicate ±s.d. between duplicates.

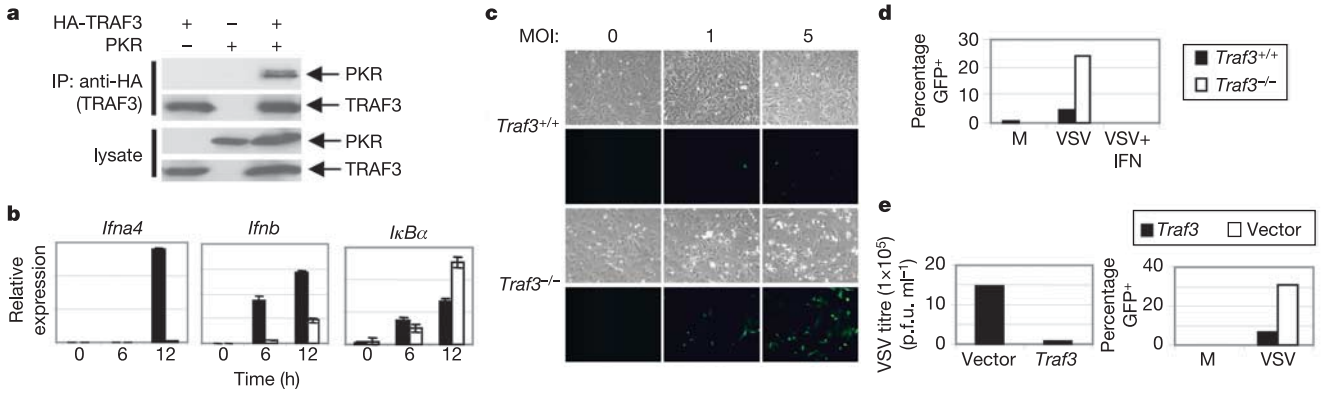

Figure 4 | Involvement of TRAF3 in TLR-independent antiviral responses. **a**, TRAF3 associates with PKR when co-expressed in 293T cells by immunoprecipitation assay. **b**, MEFs were infected with VSV and analysed for expression of the indicated genes by Q-PCR. *Traf3*$^{+/+}$, filled bars; *Traf3*$^{-/-}$, empty bars. Error bars indicate ±s.d. between duplicates. **c**, **d**, MEFs were infected with VSV expressing GFP (multiplicity of infection of 1) in the presence or absence of IFN-β (100 U ml^{-1}). Twenty-four hours later, GFP expression was visualized by fluorescent microscopy and quantified by flow cytometry. **e**, *Traf3*$^{-/-}$ MEFs were reconstituted with TRAF3, infected with VSV, and viral replication was quantified by plaque assays and flow cytometry. TRAF3 expression in reconstituted cells was confirmed by immunoblotting (data not shown).

activation of another downstream IRF7 kinase (Supplementary Fig. 1).

Defects in TLR-mediated type I IFN induction in TRAF3-deficient cells prompted us to investigate whether TRAF3 is also involved in viral-mediated type I IFN production. To address this, *Traf3*$^{+/+}$ and *Traf3*$^{-/-}$ Flt3-ligand-derived dendritic cells were infected with VSV. As shown in Fig. 3f, *Traf3*$^{-/-}$ Flt3-ligand-derived dendritic cells were almost completely defective in the production of IFN-α in response to VSV. Induction of type I IFNs in pDCs by VSV is thought to proceed through a TLR7- and MyD88-dependent pathway[10]. In contrast to pDCs, murine embryonic fibroblasts (MEFs) are thought to detect viral infection through cytoplasmic receptors such as PKR and RIG-I (refs 2, 27). PKR, a putative intracellular viral receptor, has a critical role in the induction of type I IFNs and antiviral responses to VSV infection in both MEFs and animal models[2,28]. Interestingly, PKR has been shown to activate NF-κB through a direct interaction with TRAF2 and TRAF5 (ref. 29). We found that PKR also associates with TRAF3 when expressed in 293T cells (Fig. 4a), suggesting a possible role for TRAF3 in PKR-dependent IFN production. In fact, type I IFN induction was defective in TRAF3-deficient MEFs in response to VSV infection (Fig. 4b). Accordingly, we observed increased susceptibility to VSV infection in TRAF3-deficient MEFs compared to wild-type controls (Fig. 4c, d). This defect is due to the loss of TRAF3 expression, as ectopically expressed TRAF3 in *Traf3*$^{-/-}$ cells rescued their resistance to VSV infection (Fig. 4e).

Our findings indicate that TRAF3 is required for type I IFN production in response to both TLR activation and direct viral infection (Supplementary Fig. S1). Furthermore, we provide evidence that TRAF3 can associate with putative intracellular viral receptors, such as PKR, as well as TLR adaptors and downstream IRF3/7 kinases. These findings suggest that multiple TLR-dependent and TLR-independent pathways may converge on a common TRAF3 complex involved in the regulation of type I IFN production. Thus, we show that TRAF3 is a novel critical mediator of innate antiviral responses.

METHODS

Mice. C57BL/6 (Jackson Laboratories) mice aged 6–12 weeks were used as recipients in fetal liver transplantation experiments. Targeted disruption of the *Traf3* gene and the fetal liver reconstitutions have been described previously[15]. To obtain *Traf3*$^{+/+}$ and *Traf3*$^{-/-}$*p100*$^{-/-}$ cells, *Traf3*$^{+/-}$ mice were crossed with *NF-κB p100*$^{-/-}$ mice. *Traf3*$^{-/-}$*p100*$^{-/-}$ progeny live into adulthood (J. He *et al.*, manuscript in preparation). All mice were maintained and bred under SPF conditions in the UCLA Life Sciences mouse facility. Bone marrow was harvested from reconstituted mice 6–8 weeks after reconstitution or from 8-day-old *Traf3*$^{+/+}$ and *Traf3*$^{-/-}$ mouse pups and used for the generation of BMMs or Flt3-ligand-derived dendritic cells.

Reagents. Flt3-ligand-derived dendritic cells and purified pDCs were stimulated with CpG D19 (ggTGCATCGATGCAgggggG, where upper- and lower-case letters indicate bases with phosphodiester and phosphorothioate-modified backbones, respectively)[6]. BMMs were stimulated with the phosphorothioate-modified CpG sequence TCCATGACGTTCCTGACGTT (0.1 μM). TBK1 and IKK-ε constructs were described previously[30]. Full-length *Trif* was cloned from a murine complementary DNA library into an Myc-pCMV eukaryotic expression vector. Full-length *Irak1* and *Pkr* were cloned from a human cDNA library into a pcDNA3 vector.

Viral inhibition assays. Viral inhibition experiments were performed as described previously[11].

Quantitative PCR (Q-PCR). Q-PCR analyses were done using an iCycler thermocycler (Bio-Rad) as previously described[11]. Primer sequences for *Ifna5*, *Ifnb*, *Mx1*, *IκBα*, *Isg15*, *Ifi204*, *L32*, *RANTES* (also known as *Ccl5*) and *IP10* are the same as those previously published[11,30]. Primers for *KC*, *Irf3*, *Il12* and *Ifna4* are as follows: *KC*, forward 5′-CACTGCACCCAAACCGAAGT-3′, reverse 5′-GGACAATTTTCTGAACCAAGGG-3′; *Irf3*, forward 5′-ACATCTCCAA CAGCCAGCCTAT-3′, reverse 5′-AGTCCATGTCCTCCACCAAGTC-3′; *Il12*, forward 5′-AAGTATTCAGTGTCCTGCCAGGA-3′, reverse 5′-TGCTTCC AACGCCAGTTCA-3′; *Ifna4*, forward 5′-CCTGTGTGATGCAGGAACC-3′, reverse 5′-TCACCTCCCAGGCACAGA-3′. *L32* expression measurements were conducted in tandem with the gene of interest. All data are presented as relative expression units after normalization to the average *L32* value.

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Supplementary Information is linked to the online version of the paper at www.nature.com/nature.

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