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Modulation of experimental autoimmune encephalomyelitis through TRAF3-mediated suppression of interleukin 17 receptor signaling

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Interleukin 17 (IL-17) plays critical roles in the pathogenesis of various autoimmune diseases, including experimental autoimmune encephalomyelitis (EAE). How the signals triggered by this powerful inflammatory cytokine are controlled to avoid abnormal inflammatory responses is not well understood. In this study, we report that TRAF3 is a receptor proximal negative regulator of IL-17 receptor (IL-17R) signaling. TRAF3 greatly suppressed IL-17–induced NF-κB and mitogen-activated protein kinase activation and subsequent production of inflammatory cytokines and chemokines. Mechanistically, the binding of TRAF3 to IL-17R interfered with the formation of the receptor signaling activation complex IL-17R–Act1–TRAF6, resulting in suppression of downstream signaling. TRAF3 markedly inhibited IL-17–induced expression of inflammatory cytokine and chemokine genes in vivo and consequently delayed the onset and greatly reduced the incidence and severity of EAE. Thus, TRAF3 is a negative regulator of IL-17R proximal signaling.

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Abbreviations used: BAFF, B cell–activating factor; CIA, collagen-induced arthritis; CNS, central nervous system; EAE, experimental autoimmune encephalomyelitis; ERK, extracellular signal-regulated kinase; FLS, fibroblast-like synoviocyte; JNK, c-Jun N-terminal kinase; MAPK, mitogen-activated protein kinase; MEF, mouse embryonic fibroblast; MMP, matrix metalloproteinase; MOG, myelin oligodendrocyte glycoprotein; mRNA, messenger RNA; MS, multiple sclerosis; RNAi, RNA interference; siRNA, small interfering RNA; TLR, Toll-like receptor; TRAF, TNF receptor–associated factor.

Th17 cells are a newly identified T cell subset that have a specific differentiation program different from traditional Th1 and Th2 cell subsets. The cytokines TGF-β, IL-6, IL-1, IL-23, and IL-21 are important for the differentiation and maintenance of the Th17 lineage (Bettelli et al., 2007; Ivanov et al., 2007; McGeachy and Cua, 2008; Ouyang et al., 2008; O'Shea et al., 2009). Th17 cells express and secret the signature cytokine IL-17. IL-17, also called IL-17A, is the most studied member of the IL-17 family, consisting of six cytokines (IL-17A to IL-17F; Moseley et al., 2003; Kolls and Lindén, 2004; Gaffen, 2009). It has been clearly shown that IL-17 is a major inflammation-driving cytokine, exerting its functions through inducing and sustaining the production of inflammatory cytokines, chemokines, and matrix metalloproteinases (MMPs; Ye et al., 2001). IL-17 can also act synergistically with IL-1 or TNF for further induction of proinflammatory genes (Ruddy et al., 2004; Shen et al., 2005).

Both IL-17 and Th17 cells have been found to contribute to the pathogenesis of many inflammatory autoimmune disorders in mouse models, including experimental autoimmune encephalomyelitis (EAE), collagen-induced arthritis (CIA), and inflammatory bowel disease (Nakae et al., 2003; Komiyama et al., 2006; Sato et al., 2006; Ogura et al., 2008; Chiang et al., 2009; Gaffen, 2009; Korn et al., 2009; Reboldi et al., 2009). EAE is a well characterized mouse model for human multiple sclerosis (MS). It is induced by immunization with myelin antigens such as myelin oligodendrocyte glycoprotein (MOG; MOG [35–55]) in adjuvant or by adoptive transfer of myelin-specific T cells, resulting in inflammatory infiltrates and demyelination in the central nervous system (CNS)

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and consequently axonal pathology resembling MS (Stromnes and Goverman, 2006). IL-17– or IL-17 receptor (IL-17R)–deficient mice are shown to be resistant to MOG-induced EAE (Komiyama et al., 2006; Gonzalez-García et al., 2009). Although IL-17 is found to be elevated in human patients with autoimmune diseases like MS (Lock et al., 2002), IL-17 blocking antibody can efficiently reduce autoimmune pathology in the mouse model of EAE (Park et al., 2005). These studies suggest that IL-17 plays critical roles in the pathogenesis of MS or EAE, and targeting IL-17 signaling can potentially be a powerful strategy to cure autoimmune diseases.

IL-17 has been shown to activate many common downstream signaling pathways, such as NF-κB and mitogen-activated protein kinases (MAPKs; c-Jun N-terminal kinase [JNK], P38, and extracellular signal-regulated kinase [ERK]; Laan et al., 2001; Kolls and Lindén, 2004; Gaffen, 2008; Ouyang et al., 2008). TRAF6 was shown to be required for IL-17–induced NF-κB and JNK activation. However, its in vivo function in IL-17–mediated signaling has still not been identified because of the embryonic lethal phenotype of TRAF6-deficient mice (Schwandner et al., 2000). Although IL-17R does not consist of an obvious TRAF6-binding site, structural analysis shows it contains a conserved sequence segment called SEFIR (SEF and IL-17R), which is similar to the TIR (Toll–IL-1 receptor) domain conserved in Toll/IL-1R receptors (Novatchkova et al., 2003). Interestingly, the adaptor Act1 was found to have the SEFIR domain. We and others discovered that Act1 is required for IL-17–mediated signaling and induction of downstream genes (Chang et al., 2006; Qian et al., 2007). We also found that Act1-deficient mice showed resistance to MOG-induced EAE and dextran sodium sulfate–induced colitis, supporting the essential role of Act1 in IL-17 signaling in vivo (Hunter, 2007; Lindén, 2007; Qian et al., 2007). Interestingly, although IL-17 activates the ERK pathway for downstream gene induction (Laan et al., 2001; Sebkova et al., 2004), IL-17–mediated ERK activation can also phosphorylate and inactivate the transcription factor C/EBP-β for feedback control (Shen et al., 2009). However, how IL-17–mediated signaling is negatively regulated is still largely unknown.

TRAF3 is an important negative regulator in TNF family receptors like CD40, B cell–activating factor (BAFF) receptor, and lymphotoxin β receptor (Cheng et al., 1995; VanArsdale et al., 1997; He et al., 2006; Xie et al., 2007; Gardam et al., 2008). Mechanistically, TRAF3 associates with NIK (NF-κB–inducing kinase) kinase and mediates its degradation through the TRAF2–TRAF3–cIAP1–cIAP2 complex in control of p100 processing to p52 to suppress B cell survival and immune responses in BAFF- and CD40-mediated pathways (Matsuzawa et al., 2008; Vallabhapurapu et al., 2008; Zarnegar et al., 2008). However, TRAF3 is essential for oncogene LMP1-mediated signaling, which mimics constitutive CD40 signaling (Xie et al., 2004). TRAF3-deficient mice are perinatal lethal, which can be rescued by p100 deficiency (He et al., 2006). Interestingly, TRAF3 was also found to be commonly required for Toll-like receptors (TLRs) and RIG-I–mediated type I IFN production for antiviral defense (Häcker et al., 2006; Oganesyan et al., 2006; Tseng et al., 2010). Thus, TRAF3 exerts diverse functions via different signaling pathways.

Because IL-17 is a powerful proinflammatory cytokine involved in the pathogenesis of a variety of inflammatory autoimmune diseases, uncontrolled signaling of IL-17 can potentially lead to inflammatory pathology. Although recent investigations have begun to dissect the positive signaling (Schwandner et al., 2000; Chang et al., 2006; Toy et al., 2006; Huang et al., 2007; Qian et al., 2007; Liu et al., 2009), the negative regulation of IL-17R–mediated signaling remains unclear. In this study, we demonstrate that TRAF3 is a crucial negative regulator of IL-17R–mediated signaling. TRAF3 interacts with IL-17R in a signal-dependant way. Binding of TRAF3 to IL-17R interferes with the formation of the positive signaling complex IL-17R–Act1–TRAF6, resulting in down-regulation of IL-17R–mediated signaling and suppression of IL-17–induced expression of downstream inflammatory genes. TRAF3 also controls IL-17–mediated induction of inflammatory genes in vivo and consequently the development of the autoimmune disease EAE. Our results identify TRAF3 as the first receptor proximal negative regulator in IL-17 signaling and present TRAF3 as a potential novel target for intervention of IL-17–dependant autoimmune diseases.

RESULTS

TRAF3 negatively regulates IL-17–mediated signaling

TRAF3 and TRAF6 are important signaling adaptors in many signaling pathways such as TNF superfamily receptors and IL-1/TLRs (Chung et al., 2002; Jabara et al., 2002; Pineda et al., 2007). Previous experiments have shown that TRAF6 is required for IL-17–mediated NF-κB and JNK activation (Schwandner et al., 2000). We tested whether TRAF3 has any function in IL-17–mediated pathways. First, we transfected TRAF3 or empty vector plasmids into HeLa cells and checked IL-17–mediated immediate signaling. Interestingly, we found that overexpression of TRAF3 suppressed IL-17–induced IκBα phosphorylation and degradation, p65 phosphorylation, p38 phosphorylation, and ERK phosphorylation, indicating that TRAF3 has general inhibitory effects on IL-17–mediated pathways (Fig. 1 A). We then confirmed that through the small interfering RNA (siRNA) knockdown approach. HeLa cells were infected with control siRNA or TRAF3-specific siRNA lentivirus. siTRAF3 efficiently knocked down TRAF3 expression. Compared with control siRNA, TRAF3 knockdown significantly enhanced IL-17–mediated signaling, which is consistent with the results from TRAF3 overexpression (Fig. 1 B). To further confirm this, we moved to check the function of mouse TRAF3. To avoid the variation of different mouse embryonic fibroblasts (MEFs), we put TRAF3 back into TRAF3-deficient MEFs through a mouse stem cell virus retroviral system. Restoration of TRAF3 greatly inhibited IL-17–mediated signaling (Fig. 1 C). Collectively, these data strongly

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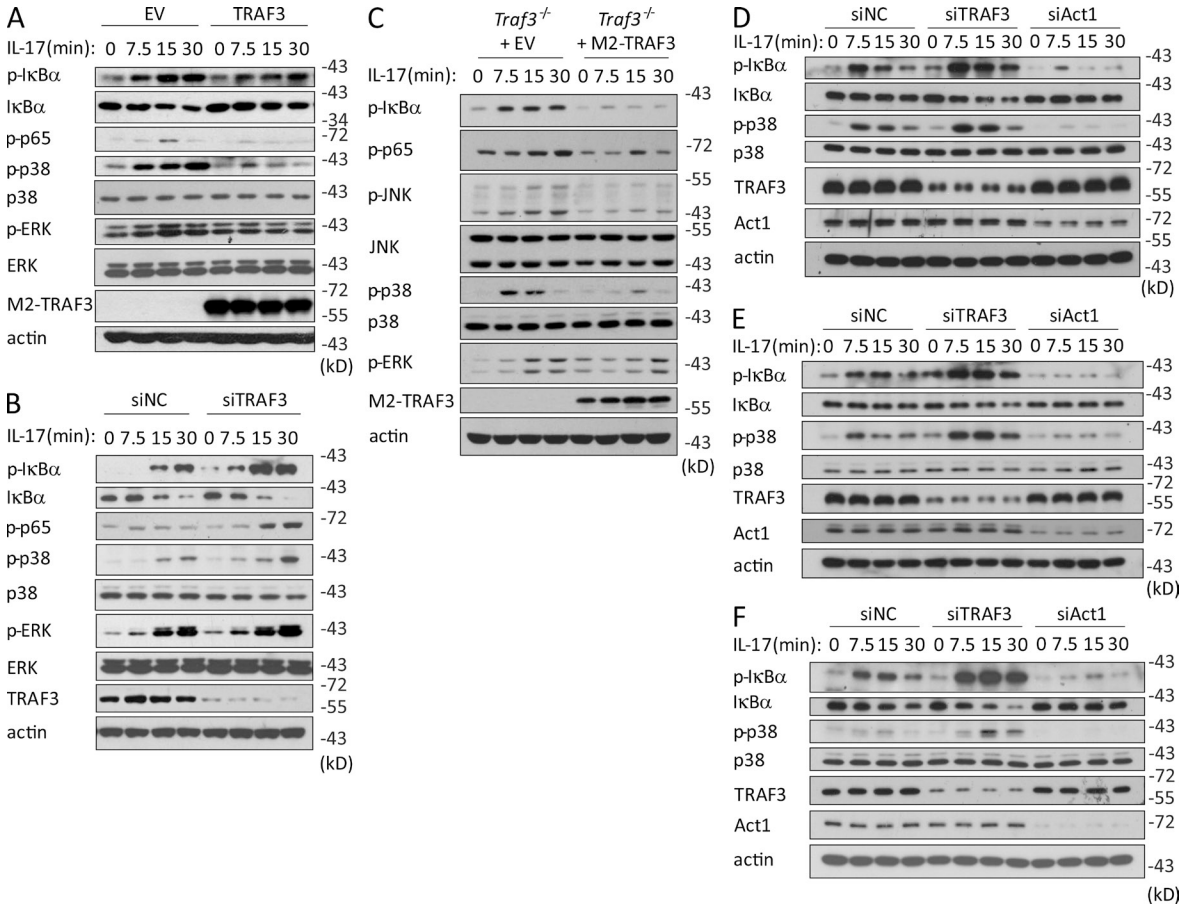

Figure 1. TRAF3 negatively regulates IL-17–mediated signaling. (A and B) HeLa cells transfected with plasmids for empty vector (EV) or M2 (Flag)-tagged TRAF3 (A) or infected with lentivirus encoding scrambled siRNA (siNC) or TRAF3 siRNA (siTRAF3; B) were left untreated or treated with IL-17 for 7.5, 15, or 30 min. Whole cell lysates were immunoblotted with anti–p-IκBα, anti-IκBα, anti–p-p65, anti–p-p38, anti-p38, anti–p-ERK, anti-ERK, anti-M2, anti-TRAF3, or anti–β-actin. (C) *Traf3*$^{-/-}$ MEFs transduced with control retrovirus or retrovirus encoding mouse TRAF3 (M2-TRAF3) were left untreated or treated with IL-17 for 7.5, 15, or 30 min. Whole cell lysates were immunoblotted with the indicated antibodies. (D–F) Mouse primary astrocytes (D), U87-MG astrocyte cell line (E), or human primary synoviocytes (FLS; F) infected with lentivirus encoding scrambled siRNA, TRAF3 siRNA, or Act1 siRNA (siAct1) were left untreated or treated with IL-17 for 7.5, 15, or 30 min. Whole cell lysates were immunoblotted with the indicated antibodies. Data are representative of three (A–C) or two (D–F) independent experiments.

suggest that TRAF3 plays a general inhibitory role in IL-17–mediated pathways.

IL-17 has been shown to play a critical role in the pathogenesis of autoimmune diseases in mouse models. We wanted to know whether endogenous TRAF3 regulates IL-17R signaling in physiologically relevant cell types. Astrocytes are reported to be the critical cell type for IL-17 signaling in mediating EAE development (Kang et al., 2010). We isolated mouse primary astrocytes and performed RNA interference (RNAi)–mediated knockdown of mouse TRAF3 through lentiviral system. We found that knockdown of TRAF3 greatly enhanced IL-17 signaling, whereas knockdown of Act1 suppressed the signaling in the primary astrocytes (Fig. 1 D). We also performed RNAi-mediated knockdown of TRAF3 in the human astrocyte cell line U87-MG and got the same results (Fig. 1 E). As IL-17 has also been known to contribute to the pathogenesis of CIA, a mouse model for human rheumatoid arthritis, we checked the function of TRAF3 in IL-17 signaling in human primary fibroblast-like synoviocytes (FLSs), an important cell type for the pathology of rheumatoid arthritis. Similarly, we found that knockdown of TRAF3 increased IL-17–mediated signaling in FLSs (Fig. 1 F). Collectively, our data show that endogenous TRAF3 plays a general inhibitory role in IL-17 signaling in both cell lines and primary cells.

TRAF3 inhibits IL-17–induced expression of inflammatory genes

The aforementioned results demonstrated that TRAF3 repressed IL-17–mediated immediate signaling. To explore the role of TRAF3 in IL-17–dependant gene transcription, we checked transcription factor NF-κB activity by luciferase assay as an example. HeLa cells were first infected with lentivirus encoding GFP and TRAF3 siRNA to knockdown

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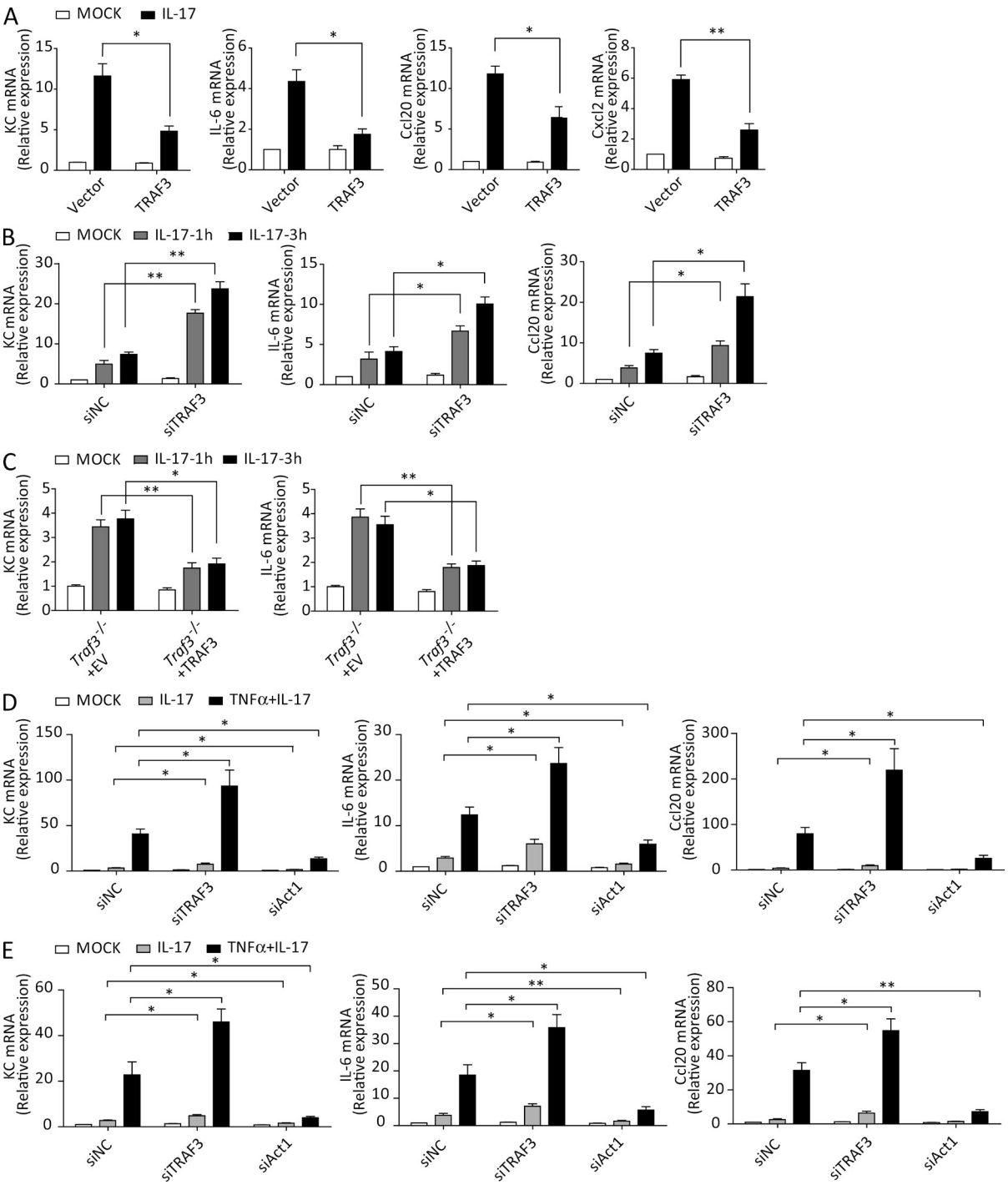

Figure 2. TRAF3 inhibits IL-17–induced expression of inflammatory cytokines and chemokines. (A and B) HeLa cells were transfected with plasmids encoding empty vector or TRAF3 (A) or infected with lentivirus encoding scrambled siRNA (siNC) or TRAF3 siRNA (siTRAF3; B) and then left untreated (mock) or stimulated with IL-17 for 1 or 3 h. The induction of *KC*, *IL-6*, *Cxcl2*, and *Ccl20* mRNA was analyzed by real-time PCR. (C) *Traf3*$^{-/-}$ MEFs transduced with control retrovirus or retrovirus encoding mouse TRAF3 were left untreated (mock) or stimulated with IL-17 for 1 and 3 h. The induction of *KC* and *IL-6* mRNA was measured by real-time PCR. (D and E) Mouse primary astrocytes (D) or human astrocyte cell line U87-MG (E) was infected with lentivirus encoding scrambled siRNA, TRAF3 siRNA, or Act1 siRNA (siAct1) and were left untreated (mock) or treated with IL-17 alone or IL-17 plus 10 ng/ml TNF. The expression of *KC*, *IL-6*, and *Ccl20* mRNA was analyzed by real-time PCR. All gene expression above was shown as fold of induction relative to that of the untreated cells. *, $P < 0.05$; and **, $P < 0.01$ (Student's *t* test). Data are representative of three (A–E) independent experiments (mean and SEM).