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

Shu Zhu,[1] Wen Pan,[1] Peiqing Shi,[1] Hanchao Gao,[1] Fang Zhao,[1] Xinyang Song,[1] Yan Liu,[1] Lihua Zhao,[1] Xiaoxia Li,[2] Yufang Shi,[1] and Youcun Qian[1]

[1]The Key Laboratory of Stem Cell Biology, Institute of Health Sciences, Shanghai Institutes for Biological Sciences, Chinese Academy of Sciences and Shanghai Jiao Tong University School of Medicine, Shanghai 200025, China
[2]Department of Immunology, Lerner Research Institute, Cleveland Clinic, Cleveland, OH 44195

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.

CORRESPONDENCE
Youcun Qian:
ycqian@sibs.ac.cn

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)

The Rockefeller University Press $30.00
J. Exp. Med. Vol. 207 No. 12 2647-2662
www.jem.org/cgi/doi/10.1084/jem.20100703

The Journal of Experimental Medicine

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

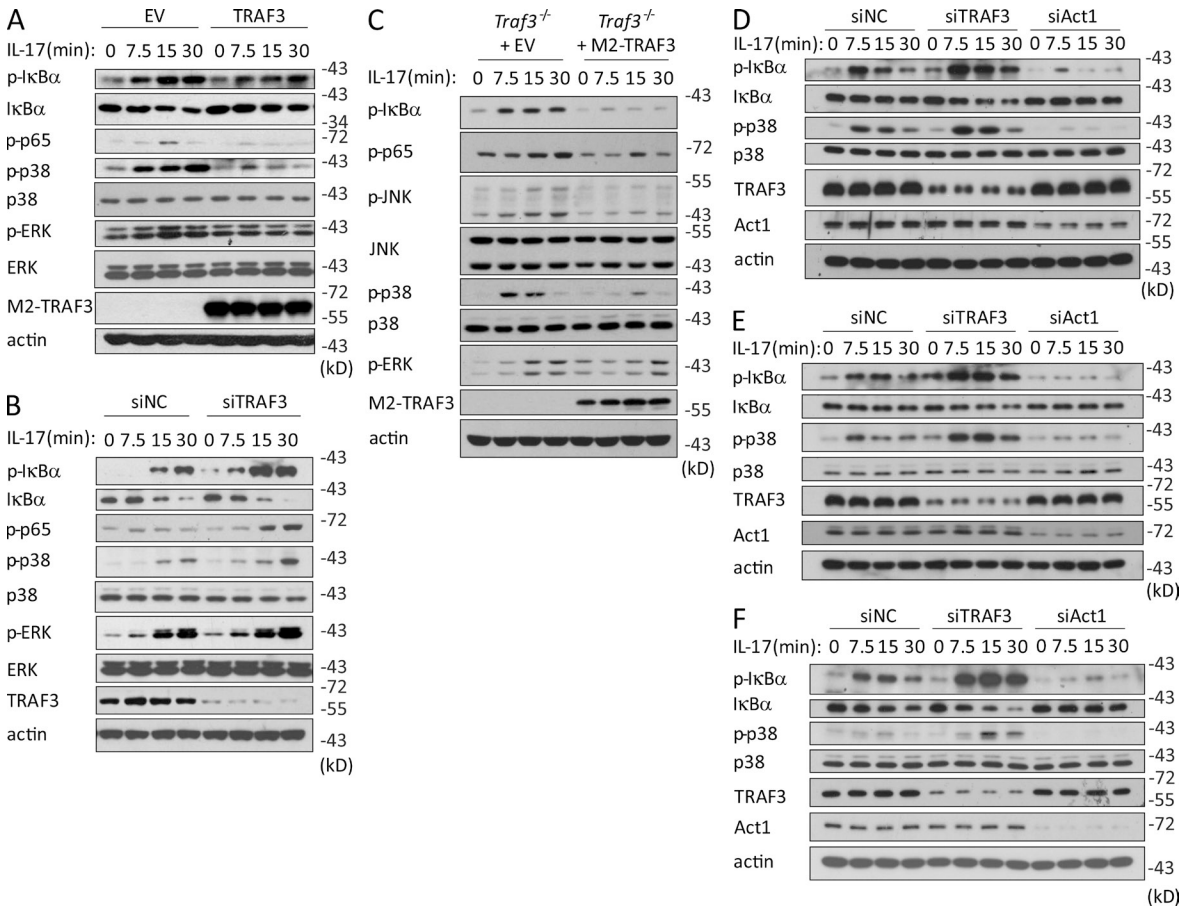

Figure 1. TRAF3 negatively regulates IL-17–mediated signaling. (A and B) HeLa cells transfected with plasmids for empty vector (EV) or M2 (Flag)-tagged TRAF3 (A) or infected with lentivirus encoding scrambled siRNA (siNC) or TRAF3 siRNA (siTRAF3; B) were left untreated or treated with IL-17 for 7.5, 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

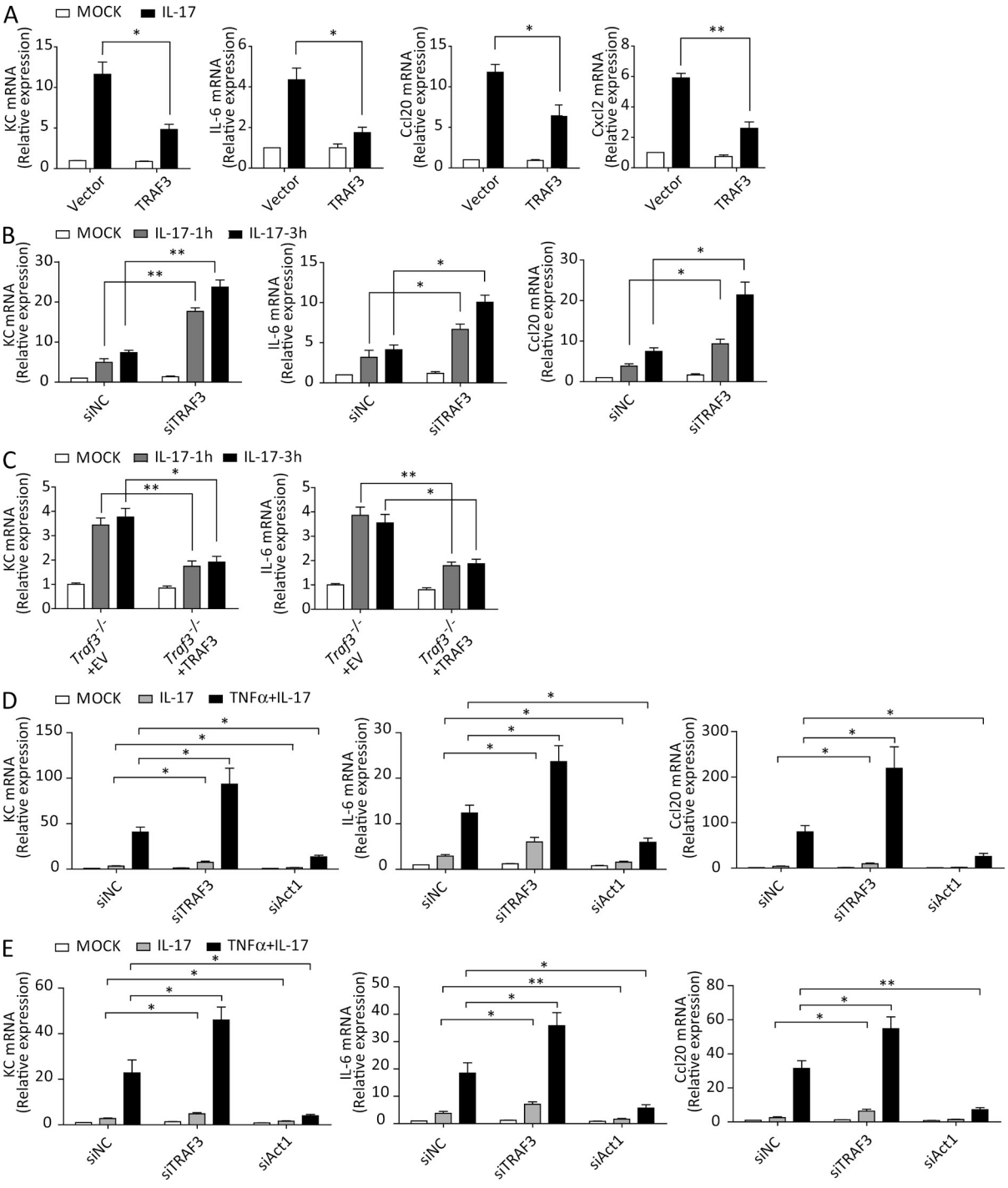

Figure 2. TRAF3 inhibits IL-17–induced expression of inflammatory cytokines and chemokines. (A and B) HeLa cells were transfected with plasmids encoding empty vector or TRAF3 (A) or infected with lentivirus encoding scrambled siRNA (siNC) or TRAF3 siRNA (siTRAF3; B) and then left untreated (mock) or 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).

TRAF3 expression. GFP-positive cells were then sorted out by flow cytometry and transfected with NF-κB luciferase construct. After transduction, the cells were stimulated with or without IL-17. Knockdown of TRAF3 significantly increased IL-17–mediated NF-κB luciferase activity (Fig. S1 A). Similarly, TRAF3-deficient MEFs showed much higher NF-κB luciferase activity than WT MEFs, whereas TRAF3 restoration into TRAF3-deficient MEFs significantly suppressed IL-17–induced NF-κB luciferase activity (Fig. S1 B). Together, these results indicate that TRAF3 plays an important inhibitory role in IL-17–mediated NF-κB activation and transcription.

To further confirm the negative role of TRAF3 in IL-17–mediated pathways, we checked the expression of IL-17–induced genes. Although IL-17 strongly induced the expression of inflammatory cytokines or chemokines, including *KC*, *IL-6*, *Cxcl2*, and *Ccl20* in HeLa cells, TRAF3 overexpression dramatically suppressed IL-17–induced expression of these genes (Fig. 2 A). On the contrary, knockdown of TRAF3 in HeLa cells significantly increased IL-17–mediated gene expression (Fig. 2 B and Fig. S1 C). The inhibitory phenomenon of TRAF3 was also found in the mouse cell system. TRAF3 restoration into TRAF3-deficient MEFs greatly reduced the IL-17–induced gene expression (Fig. 2 C). We also investigated the function of endogenous TRAF3 in regulating IL-17–induced production of proinflammatory genes in the physiological relevant cell types like astrocytes and synoviocytes. We observed that knockdown of TRAF3 in mouse primary astrocytes increased the IL-17–induced production of inflammatory genes, including *KC*, *IL-6*, and *Ccl20*, whereas knockdown of Act1 decreased the induction of those genes (Fig. 2 D). IL-17 has been known to synergize with TNF in the induction of the proinflammatory genes. Similarly, knockdown of TRAF3 increased, whereas knockdown of Act1 decreased the synergy of IL-17 and TNF in induction of the proinflammatory genes in the primary mouse astrocytes (Fig. 2 D). Similar observations were also found in human astrocytes (Fig. 2 E) and human primary synoviocytes (Fig. S1 D), in which knockdown of TRAF3 increased, whereas knockdown of Act1 decreased IL-17–mediated or the combination of IL-17– and TNF-mediated induction of the proinflammatory genes. TRAF3 also dramatically suppressed the synergy when low dose of TNF was used (Fig. S1, E and F). These data clearly show that endogenous TRAF3 negatively regulates IL-17–mediated downstream gene induction in different cell types.

IL-17 stimulation induces the recruitment of TRAF3 to IL-17R

Because our aforementioned results showed that TRAF3 plays a general inhibitory role in IL-17–mediated pathways, including NF-κB and MAPKs, we next checked whether TRAF3 functions at the receptor level through its recruitment to IL-17R upon IL-17 stimulation. To test the hypothesis, we generated HeLa cell lines stably expressing Flag-tagged IL-17R and performed coimmunoprecipitation assay in a signal-dependant way. IL-17 stimulation led to transient interaction of IL-17R with TRAF3. But the recruitment of TRAF3 to IL-17R is slower compared with that of Act1 to IL-17R, implying that IL-17R may first recruit Act1 for activation signaling and then recruit TRAF3 for timely turning off the activation signaling (Fig. 3 A).

There are five receptors, IL-17RA to IL-17RE, in the IL-17R superfamily. Because both IL-17RA and IL-17RC are required for IL-17 signaling, we wondered whether TRAF3 also binds to IL-17RC. We checked the interaction of TRAF3 with all the five receptors in 293 cells by overexpression. We found that TRAF3 indeed bound to IL-17RC, in addition to IL-17RA (Fig. S2 A). TRAF3 only weakly interacted with IL-17RE but did not associate with IL-17RB or IL-17RD. We further confirmed the interaction of TRAF3 with IL-17RC in IL-17 signaling. We generated HeLa cells stably expressing Flag-tagged IL-17RA and HA-tagged IL-17RC and performed immunoprecipitation. We found

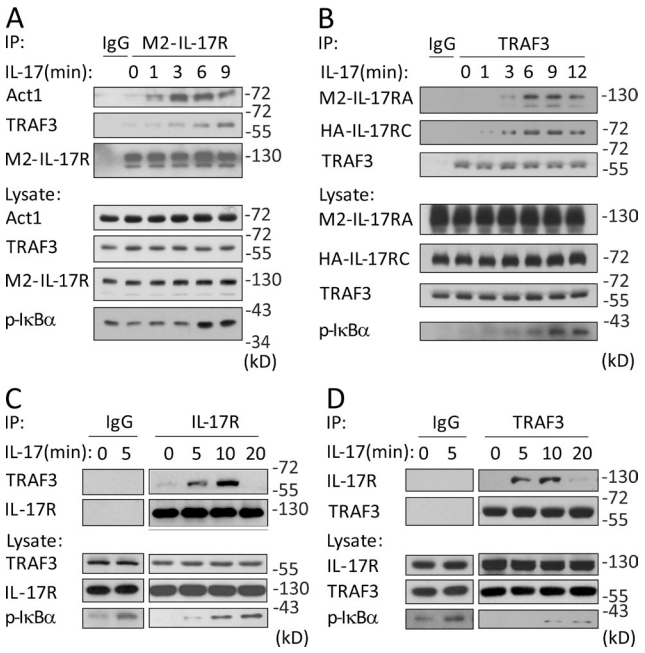

Figure 3. TRAF3 is recruited to IL-17R upon IL-17 stimulation. (A) HeLa cells stably expressing M2 (Flag)–IL-17RA were either left untreated or treated with IL-17 for 1, 3, 6, or 9 min. Whole cell lysates were immunoprecipitated (IP) with anti-M2 or control IgG and followed by immunoblotting with anti-TRAF3, anti-Act1, and anti-M2. Whole cell lysates were also immunoblotted with anti-Act1, anti-TRAF3, or anti-M2 to check the expression level of individual proteins or with anti–p-IκBα to check IL-17 responsiveness. (B) HeLa cells stably expressing M2–IL-17RA and HA–IL-17RC were either left untreated or treated with IL-17 for 1, 3, 6, 9, or 12 min. Whole cell lysates were immunoprecipitated with anti-TRAF3 and followed by immunoblotting with anti-M2, anti-HA, and anti-TRAF3. (C and D) HeLa cells were left untreated or treated with IL-17 for 5, 10, or 20 min. Whole cell lysates were immunoprecipitated with IgG or anti–IL-17R (C) or IgG or anti-TRAF3 (D) and followed by immunoblotting with anti-TRAF3 or anti–IL-17R. Data are representative of three (A, C, and D) or two (B) independent experiments.

that IL-17 stimulation led to signal-dependent association of TRAF3 with IL-17RC, similar to the interaction of TRAF3 with IL-17RA (Fig. 3 B). These data suggest that TRAF3 binds to IL-17RA–IL-17RC heterodimers to inhibit IL-17 signaling.

To avoid the overexpression system, we further tested the interaction of endogenous IL-17R and TRAF3 in HeLa cells in IL-17 signaling. Whole cell lysates were immunoprecipitated with anti–IL-17R or control IgG and Western blotted with anti-TRAF3. Consistent with the results from the overexpression system, IL-17 stimulation also led to transient association of IL-17R with TRAF3 (Fig. 3 C). We confirmed that the IL-17R antibody did not cross react with IL-17RC (Fig. S2 B). We next immunoprecipitated TRAF3 with anti-TRAF3 or control IgG and checked the binding protein with IL-17R antibody. Similar results were found that TRAF3 interacted with IL-17R in an IL-17 signal–dependant way (Fig. 3 D). Because IL-17 induced the recruitment of TRAF3 to IL-17R, we looked for the potential TRAF3-binding domains or sites in the IL-17R. The cytoplasmic part of IL-17R has been reported to consist of SEFIR, TILL (TIR-like loop), and distal domains with different functions (Fig. S3 A; Maitra et al., 2007). Through coimmunoprecipitation in HEK293 cells, TRAF3 was shown to strongly interact with IL-17R upon overexpression, as we previously observed (Qian et al., 2007). However, deletion of the distal domain of IL-17R blocked the interaction of IL-17R and TRAF3, whereas deletion of SEFIR domain or TILL domain had no effect or partial effect, respectively, indicating that TRAF3 binds to the distal domain of IL-17R (Fig. S3 B).

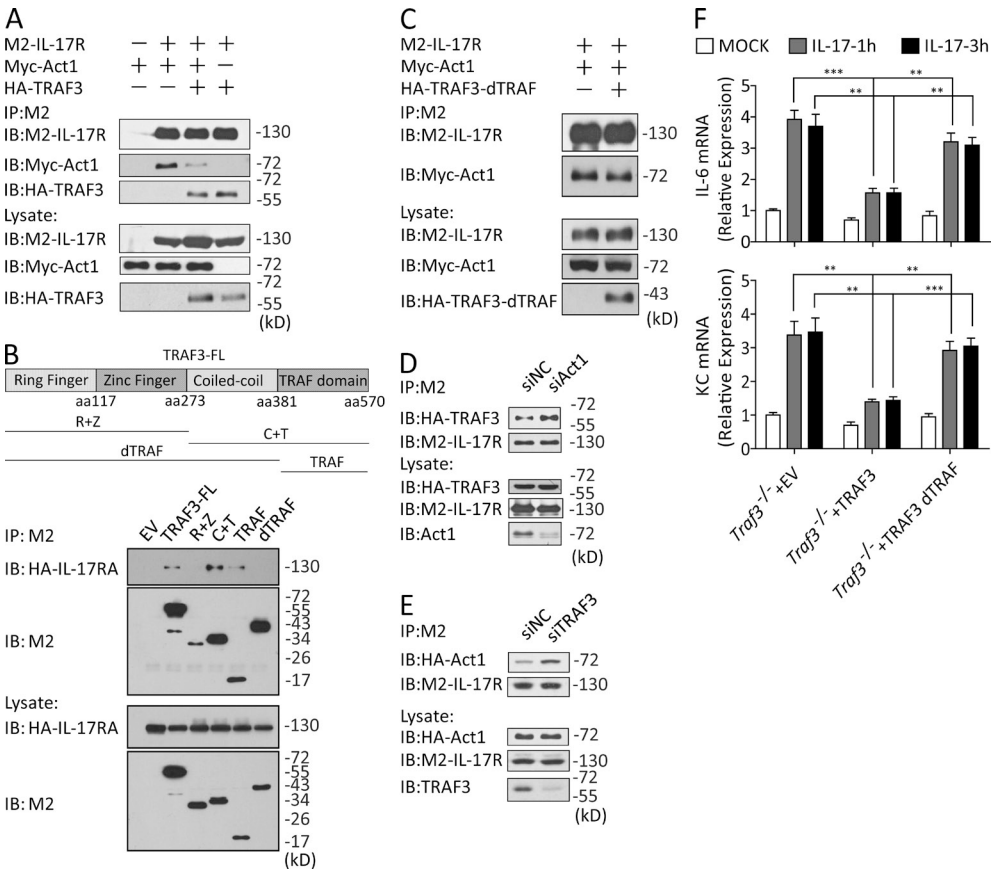

Figure 4. TRAF3 competes with Act1 to interact with IL-17R. (A) HEK293 cells were transfected with the combined plasmids as indicated. Whole cell lysates were immunoprecipitated (IP) with anti-M2, followed by immunoblotting (IB) with anti-M2, anti-Myc, or anti-HA. (B) TRAF3 deletion mutants are shown in the top panel. HEK293 cells were transfected with plasmids encoding HA-tagged IL-17R and empty vector (EV) or Flag (M2)-tagged TRAF3 or its deletion mutants. Whole cell lysates were immunoprecipitated with anti-M2 and then immunoblotted with anti-HA or anti-M2. (C) HEK293 cells were transfected with the combined plasmids as indicated. Whole cell lysates were immunoprecipitated with anti-M2, followed by immunoblotting with anti-M2 or anti-Myc. Whole cell lysates were also immunoblotted with anti-M2, anti-Myc, or anti-HA. (D and E) HEK293 cells infected with lentiviruses encoding scrambled (siNC) or Act1 (siAct1) siRNA (D) or scrambled or TRAF3 (siTRAF3) siRNA (E) were then transfected with expression plasmids encoding M2–IL-17R and HA-TRAF3 (D) or M2–IL-17R and HA-Act1 (E). Whole cell lysates were immunoprecipitated with anti-M2, followed by immunoblotting with anti-HA or anti-M2. (F) TRAF3-deficient MEFs first transduced with retrovirus encoding empty vector (EV), mouse full-length TRAF3, or TRAF3 lacking TRAF domain (TRAF3 dTRAF) were then left untreated (mock) or stimulated with IL-17 for 1 or 3 h. Induction of *KC* and *IL-6* mRNA was analyzed by real-time PCR and shown as fold of induction relative to the individual untreated MEFs. **, $P < 0.01$; and ***, $P < 0.001$ (Student's *t* test). Data are representative of three (A and C–F) or four (B) independent experiments (mean and SEM in F).

We next looked into the primary structure of IL-17R and observed two potential TRAF3-binding sites, TB1 (AXEE) and TB2 (PXEE; Fig. S3 A). To examine whether these two sites are important for the association of IL-17R to TRAF3, we point-mutated the sites and performed immunoprecipitation assay. Mutation of the TB2 site severely impaired the interaction of IL-17R and TRAF3, whereas mutation of the TB1 site had no or mild effect, suggesting that TRAF3 binds to the TB2 site of IL-17R (Fig. S3 C). All together, our data suggest that TRAF3 functions at the receptor level in regulating IL-17–mediated signaling.

The binding of TRAF3 to IL-17R interferes with the formation of the IL-17R–Act1–TRAF6 activation complex

Given the fact that both TRAF3 and Act1 bind to IL-17R but function in the opposite way, we reasoned that TRAF3 could compete with Act1 to interact with IL-17R and therefore inhibit the formation of the IL-17R–Act1 activation signaling complex. To test this hypothesis, we transfected HEK293 cells with different combinations of plasmids as indicated in Fig. 4 A and performed coimmunoprecipitation. Indeed, the addition of TRAF3 severely suppressed the interaction of IL-17R with Act1, whereas the addition of Act1 reduced the association of TRAF3 with IL-17R (Fig. 4 A). We then mapped the domains of TRAF3 to interact with IL-17R. TRAF3 contains Ring finger domain followed by zinc finger domain, coiled-coil domain, and TNF receptor–associated factor (TRAF) domain (He et al., 2007). We made different deletion constructs as shown in Fig. 4 B and performed coimmunoprecipitation to detect their interactions with IL-17R. We found that the constructs containing TRAF domain associated with IL-17R, whereas those constructs without TRAF domain did not, indicating that the TRAF domain of TRAF3 is responsible for its interaction with IL-17R (Fig. 4 B). Consistently, the TRAF domain deletion mutant of TRAF3 did not inhibit the binding of IL-17R to Act1, proving that the TRAF domain of TRAF3 associates with IL-17R to interfere with the binding of Act1 to IL-17R (Fig. 4 C). To further confirm the competitive binding hypothesis, we separately knocked down Act1 or TRAF3 and then checked protein interaction. We found that the association of TRAF3 with IL-17R was increased when Act1 expression was reduced (Fig. 4 D). Similarly, the binding of Act1 to IL-17R was enhanced when TRAF3 was knocked down (Fig. 4 E). These results strongly suggest that binding of TRAF3 to IL-17R dissociates the complex of Act1 and IL-17R.

Because TRAF6 has also been shown to be recruited to IL-17R (Schwandner et al., 2000; Qian et al., 2007), we checked whether TRAF3 also competes with TRAF6 to bind to IL-17R. We transfected HEK293 cells with different combinations of plasmids as indicated in Fig. S4 and performed coimmunoprecipitation. The addition of TRAF3 also severely suppressed the interaction of IL-17R and TRAF6 (Fig. S4 A), whereas the TRAF3 mutant with TRAF domain deleted lost its suppressive effect (Fig. S4 B). We then mapped the domains of IL-17R to interact with TRAF6. Both WT and the distal deletion mutant but not the SEF deletion mutant showed interaction with TRAF6 (Fig. S4 C), indicating that TRAF6 binds to the SEF domain of IL-17R. Because we previously found that Act1 also interacts with IL-17R through the SEF domain (Qian et al., 2007) and the SEF domain has no obvious TRAF-binding site, these results suggest that TRAF6 may associate with IL-17R through Act1.

To prove the physiological function of the competitive binding of TRAF3 to IL-17R, we made use of the TRAF domain deletion mutant of TRAF3 and performed a functional restoration assay in TRAF3-deficient MEFs (Fig. 4 F). Restoration of WT TRAF3 resulted in strong inhibition of IL-17–induced *IL-6* or *KC* production both at 1 and 3 h after stimulation. However, the deletion mutant of TRAF3, which cannot bind to IL-17R, no longer dramatically suppressed IL-17–induced gene expression of *IL-6* and *KC*, indicating that TRAF3 exerts its negative role in IL-17 signaling through its competitive binding to IL-17R. All together, our results suggest that the binding of TRAF3 to IL-17R disrupts the formation of the IL-17R–Act1–TRAF6 activation complex, resulting in suppression of IL-17–mediated signaling and induction of downstream genes.

TRAF3 represses IL-17–induced expression of inflammatory factors in vivo

To study the physiological role of TRAF3 in IL-17–mediated pathways, we generated TRAF3 transgenic mice. Five founders were obtained by genotyping through PCR (unpublished data), and two founders were characterized in more detail. The TRAF3 expression level was significantly higher in these two transgenic founders (TG1 and TG2) than that in WT controls (WT1 and WT2) in all of the tissues checked, including brain, spleen, and liver (Fig. 5 A). These two founders were therefore used for further investigations. We next generated adenovirus system for efficient delivery of virus and high expression of mouse IL-17 in mice. The IL-17 was efficiently expressed in different mouse tissues checked, including brain and spinal cord. It is worth noting that the overexpression level of IL-17 in different tissues is quite similar between TRAF3 transgenic mice and WT controls. So we continued to use the adenovirus system to overexpress IL-17 in vivo and assess the effects of TRAF3 in IL-17–mediated gene induction in vivo. IL-17 significantly induced the expression of inflammatory factors like *KC*, *IL-6*, and *MMP3* in different tissues checked, including brain and spinal cord of WT mice by real-time PCR. However, the induction of those inflammatory factors by IL-17 was dramatically suppressed in TRAF3 transgenic mice (Fig. 5 B). We next measured the protein level of IL-17, IL-6, and KC in the serum of WT and transgenic mice by ELISA. Consistently, whereas the IL-17 protein level was slightly increased in the sera of TRAF3 transgenic mice, the protein levels of IL-6 and KC were greatly reduced in the sera of TRAF3 transgenic mice compared with WT mice (Fig. 5 C). These results suggest that TRAF3 suppresses IL-17–induced expression of inflammatory factors in vivo.

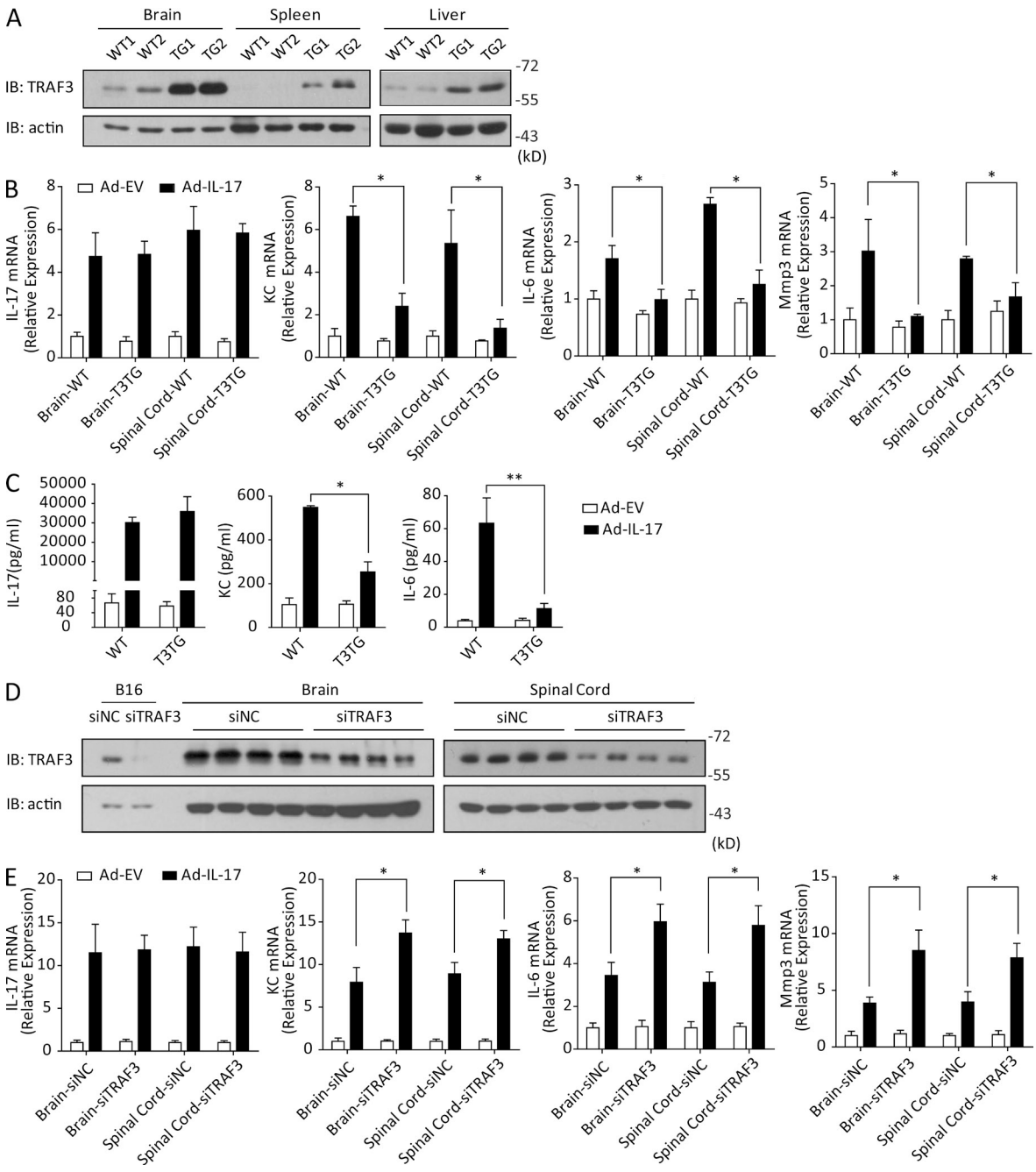

Figure 5. TRAF3 represses IL-17–induced expression of inflammatory factors in vivo. (A) TRAF3 expression was checked by immunoblotting (IB) with anti-TRAF3 from lysates of brain, spleen, and liver from two WT control mice (WT1 and WT2) or two TRAF3 transgenic founders (TG1 and TG2). (B and C) WT control or TRAF3 transgenic mice (T3TG; $n = 4$/group) were injected i.v. with adenovirus encoding empty vector (Ad-EV) or mIL-17 (Ad-IL-17) for 4 d. *IL-17*, *KC*, *IL-6*, and *Mmp3* mRNA in the brain and spinal cord was measured by real-time PCR (B), and IL-17, IL-6, and KC protein in the serum was measured by ELISA (C). (D) TRAF3 expression was checked by immunoblotting with anti-TRAF3 from lysates of B16 cells and brain and spinal cord from four pairs of mice injected intracerebroventricularly with lentivirus encoding scrambled siRNA (siNC) or TRAF3 siRNA (siTRAF3). (E) C57BL/6 mice were injected intracerebroventricularly with lentivirus encoding scrambled siRNA or TRAF3 siRNA ($n = 4$/group) for 4 d and then were injected i.v. with adenovirus encoding empty vector or mIL-17 for 4 d. *IL-17*, *KC*, *IL-6*, and *Mmp3* mRNA in the brain and spinal cord was measured by real-time PCR. mRNA expression is shown as fold of induction relative to that in the WT control mice. *, $P < 0.05$; and **, $P < 0.01$ (Student's *t* test). Data are representative of three (A–C) or two (D and E) independent experiments (mean and SEM in B, C, and E).

To confirm the results from the TRAF3 transgenic mice, we set up an RNAi approach to knock down TRAF3 in mice in vivo through lentiviral system, which has been reported to be efficient for gene knockdown in the CNS (Saijo et al., 2009; Regev et al., 2010). The lentivirus for silencing TRAF3 was concentrated and checked for knockdown efficiency (>90%) in mouse B16 cells (Fig. 5 D) and then injected intracerebroventricularly to knock down TRAF3 in the CNS in mice. We found that knockdown efficiency of TRAF3 was >50% in both brain and spinal cord (Fig. 5 D). Thus, we used this in vivo knockdown approach to study the function of TRAF3 and found that knockdown of TRAF3 in mice significantly increased IL-17–induced production of the inflammatory genes in both brain and spinal cord (Fig. 5 E), which is consistent with the results from the TRAF3 transgenic mice (Fig. 5 B).

TRAF3 critically controls EAE development

IL-17 has been shown to play critical roles in the development of autoimmune diseases like EAE. In this study, we have demonstrated that TRAF3 plays important negative roles in IL-17–mediated signaling pathways and downstream gene induction of inflammatory factors both in vitro and in vivo. We next explored whether TRAF3 modulates the development of the IL-17–mediated autoimmune disease EAE. Whereas EAE was strongly induced after MOG immunization in the WT control mice with a peak clinical score of 3.2, the disease severity was dramatically reduced in TRAF3 transgenic mice with the peak clinical score of 1.5 (Fig. 6 A). The onset of EAE induction was also significantly delayed in the TRAF3 transgenic mice, occurring at day 11 after MOG immunization, compared with WT control mice with the onset at day 7. Similarly, the EAE disease incidence (57%) was greatly reduced in the TRAF3 transgenic mice, compared with the 100% incidence in WT control mice (Fig. 6 A).

Local IL-17–mediated signaling is considered to be important for inducing plenty of inflammatory factors to recruit numerous immune cells to CNS, resulting in inflammation and tissue disruption. Consistent with the clinical scores, histological analyses by hematoxylin and eosin (H&E) and luxol fast blue staining showed that inflammatory infiltration and demyelination were much reduced in the spinal cords of TRAF3 transgenic mice after MOG immunization compared with WT mice (Fig. 6 B). Furthermore, real-time PCR analyses showed that although MOG immunization strongly induced the expression of inflammatory factors like *KC*, *Cxcl2*, *IL-6*, and *Mmp3* in the spinal cords and brains of WT mice, the induction of these inflammatory genes was dramatically decreased in the spinal cords and brains of the TRAF3 transgenic mice. These results suggest that TRAF3 controls EAE development likely through its inhibitory role in IL-17–mediated gene induction of inflammatory factors (Fig. 6 C).

We also performed EAE induction after TRAF3 was knocked down in the CNS. We observed significantly increased EAE clinic scores in the TRAF3 knockdown mice compared with control mice (Fig. 6 D). The H&E staining also showed increased infiltration of inflammatory cells in the TRAF3 knockdown mice (Fig. 6 E). Similarly, real-time analysis showed increased production of inflammatory genes during EAE induction in the TRAF3 knockdown mice (Fig. 6 F). Our results from RNAi-mediated knockdown of TRAF3 in mice in vivo are consistent with the data obtained from the TRAF3 transgenic mice showing that TRAF3 dramatically suppressed EAE development.

TRAF3-mediated suppression of IL-17 signaling in the CNS is likely responsible for its control of EAE development

To fully prove that TRAF3 in the CNS plays a major role in controlling EAE development, bone marrow transfer experiments were conducted. Both TRAF3 transgenic and WT control mice were irradiated to have their hematopoietic cells removed and then injected i.v. with WT bone marrow cells. After reconstitution, the mice were immunized with MOG (35–55) for EAE development. Similar to the results from TRAF3 transgenic mice (Fig. 6 A), the disease onset was delayed, and the severity and incidence of EAE induction was dramatically reduced in TRAF3 transgenic mice transplanted with WT bone marrow cells (Fig. 7 A), indicating that TRAF3 likely controls EAE development through its negative function in the CNS-resident cells.

Because we clearly showed that TRAF3 inhibited IL-17–mediated signaling and downstream gene induction of inflammatory factors both in vitro and in vivo (Figs. 1, 2, and 5), we next checked whether TRAF3-mediated suppression of IL-17 signaling is really responsible for the observed inhibitory effect on EAE in the TRAF3 transgenic mice. Th17 cell transfer can induce EAE, which is normally used for assessing the IL-17–mediated effect on EAE induction. We found that EAE induced by Th17 cell transfer was dramatically suppressed in the TRAF3 transgenic mice compared with control mice (Fig. 7 B), similar to MOG-induced EAE (Fig. 6 A), indicating that TRAF3-mediated suppression of IL-17 signaling is mainly responsible for its suppressive effect on EAE.

It is known that TRAF3 is essential for TLR-mediated production of IL-10 and type I IFN, which have been shown to play important inhibitory roles in EAE development (Cua et al., 2001; Fillatreau et al., 2002; Prinz et al., 2008). We next checked whether TRAF3-mediated production of IL-10 and type I IFN was involved in the observed suppressive effect of TRAF3 on EAE induction. We first assessed the expression of IL-10 and type I IFN in the TRAF3 transgenic mice during EAE induction. Although undetectable in the sera by ELISA (not depicted), *IL-10* was potently induced in the spinal cords of both TRAF3 transgenic mice and control mice (Fig. 7 C). But there was no significant difference in the induction level of *IL-10* between the transgenic and control groups. Similarly undetectable in the sera (not depicted), both *IFNα4* and *IFNβ* were also induced in the CNS during EAE, but the induction levels were much lower compared with that of IL-10 (Fig. 7 C). The induction of *IFNα4* and

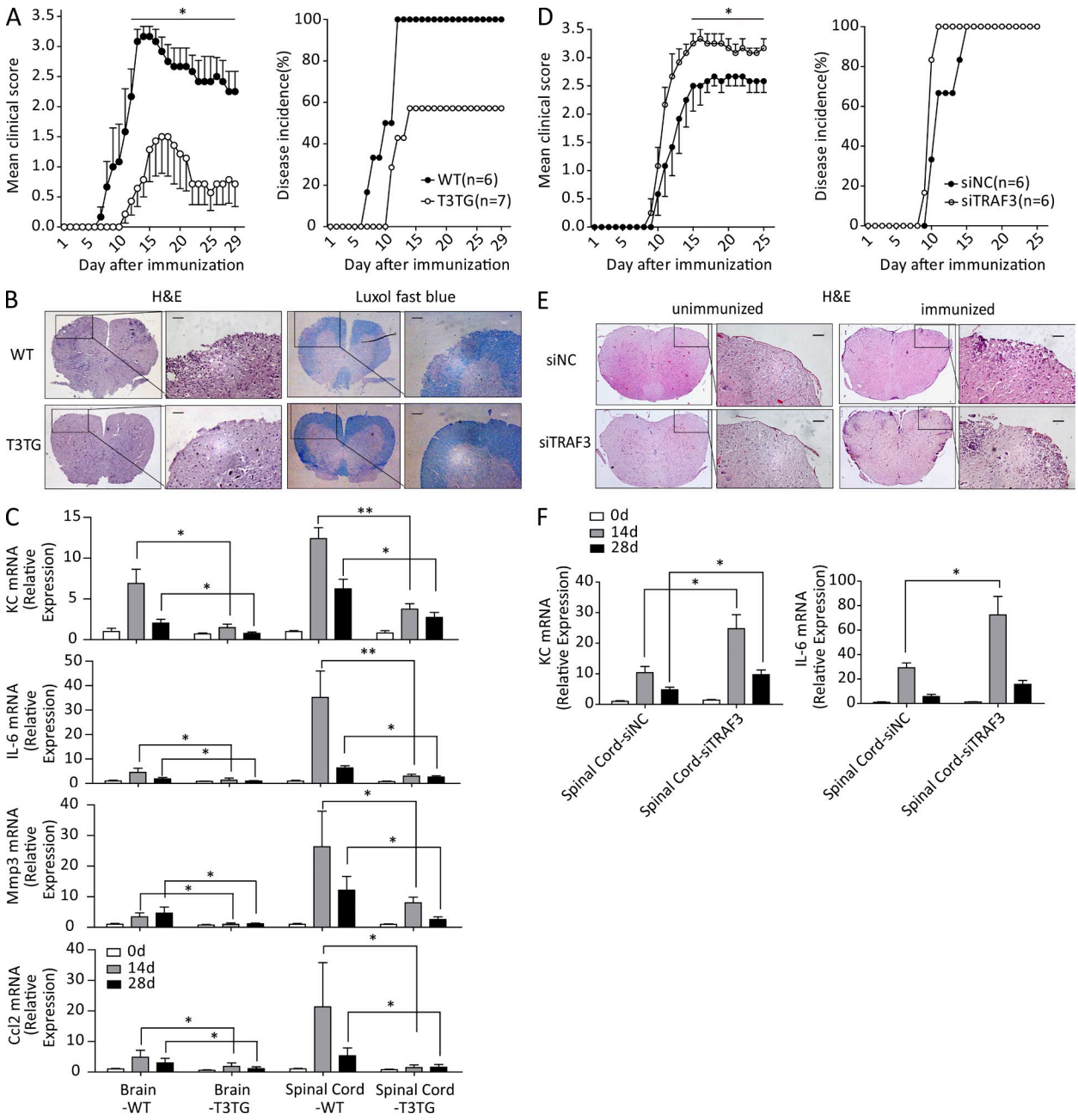

Figure 6. TRAF3 critically controls EAE development. (A–F) WT control mice or TRAF3 transgenic (T3TG) mice were immunized with MOG (35–55) to induce EAE (A–C), or WT mice were injected intracerebroventricularly with lentivirus encoding scrambled siRNA (siNC) or TRAF3 siRNA (siTRAF3; n = 5–6/group) for 4 d and then were immunized with MOG (35–55) to induce EAE (D–F). (A and D) Mean clinical scores (±SEM) and disease incidence were calculated daily according to the standards described in Materials and methods. (B and E) The spinal cords were histologically analyzed by H&E (B and E) or luxol fast blue staining (B) 16 d after immunization. The right panels show the magnifications of the boxed areas in the left panels. Bars, 50 µm. (C and F) *KC*, *IL-6*, *MMP3*, and *Ccl2* (C) or *KC* and *IL-6* mRNA (F) in the spinal cords or brains 0, 14, or 28 d after immunization was measured by real-time PCR. Expression of mRNA is shown as fold of induction relative to the untreated WT control mice. *, $P < 0.05$; and **, $P < 0.01$ (Student's t test). Data are representative of four (A), three (B and C), or two (D–F) independent experiments (mean and SEM in A, C, D, and F).

IFNβ was only slightly increased in the TRAF3 transgenic mice compared with control mice (Fig. 7 C). All together, we unexpectedly observed no big difference of *IL-10* and type I IFN production between the transgenic and control mice during EAE induction.

To really prove that the observed inhibitory effect of TRAF3 on EAE development is not mainly through IL-10 and type I IFN, we used the blocking antibodies of IL-10 and IFNAR1 and then performed EAE induction. As expected, the antibody blockage of IL-10 and IFNAR1 increased the

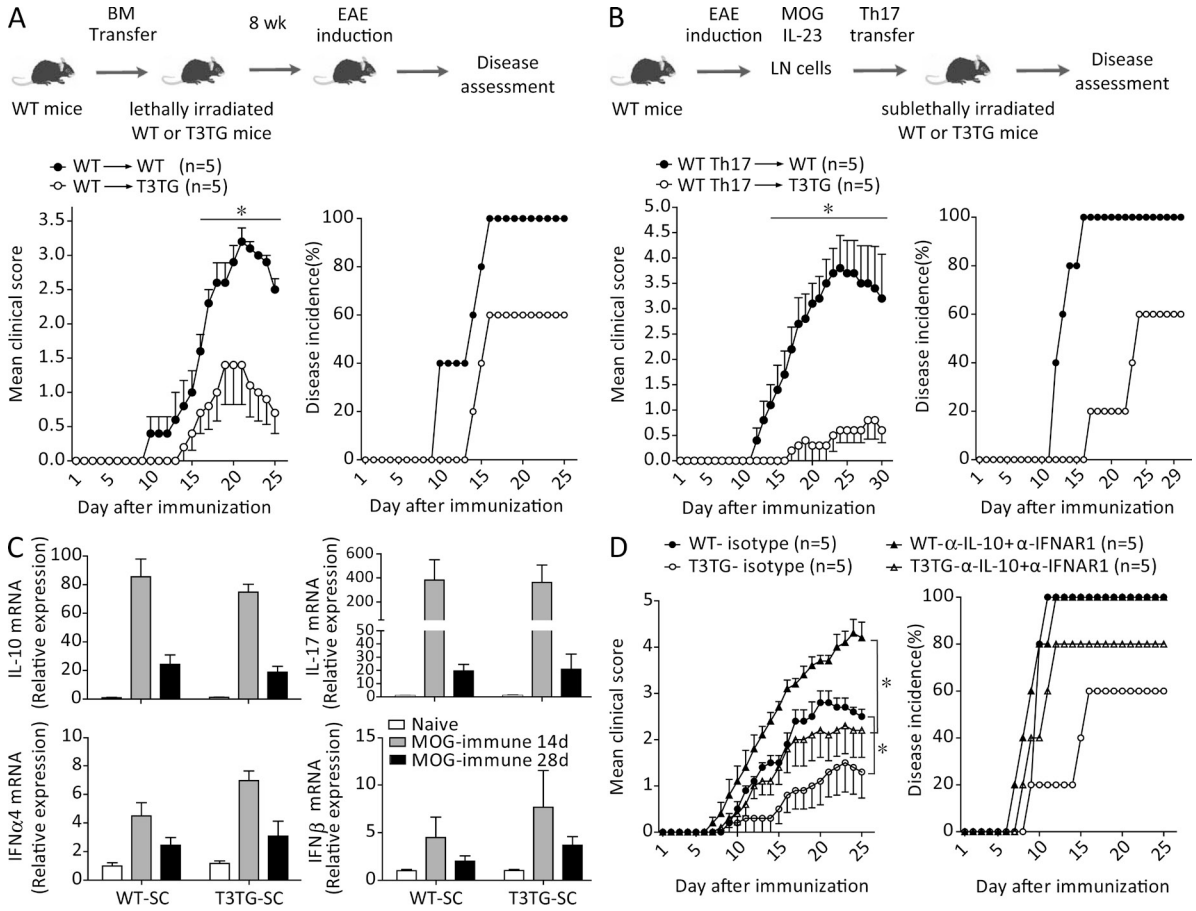

Figure 7. TRAF3 controls EAE development likely through its suppression of IL-17 signaling. (A) WT control or TRAF3 transgenic (T3TG) mice were first lethally irradiated and then reconstituted with WT bone marrow cells by i.v. injection. 8 wk after reconstitution, the mice were immunized with MOG (35–55) to induce EAE. (B) Lymphocytes from LNs of mice 10 d after MOG immunization were cultured with MOG and IL-23 for 4 d. These TH17 cells were then injected i.v. into sublethally irradiated WT or TRAF3 transgenic mice. Mean clinical scores (±SEM) and disease incidence were calculated daily. (C) *IL-10*, *IFNα4*, *IFNβ*, and *IL-17* mRNA was measured by real-time PCR in the spinal cords (SC) from TRAF3 transgenic or WT mice 14 or 28 d after MOG immunization (n = 6/group). Expression of mRNA is shown as fold of induction relative to naive, unmanipulated WT mice. (D) TRAF3 transgenic or WT mice (n = 5/group) were injected i.v. with IL-10 and IFNAR1 blocking antibodies or appropriate isotype controls, respectively at 0, 4, 8, 12, 16, or 20 d after MOG immunization. Mean clinical scores (±SEM) and disease incidence were calculated daily. *, $P < 0.05$ (Student's t test). Data are representative of three (A) or two (B–D) independent experiments.

EAE clinical scores in both TRAF3 transgenic mice and control mice (Fig. 7 D). However, under the same condition of the antibody blockage, the EAE clinical scores were dramatically reduced in the TRAF3 transgenic mice compared with control mice (Fig. 7 D), indicating that the production of IL-10 and type I IFN is not important for the suppressive effect on EAE in the TRAF3 transgenic mice. Collectively, our results suggest that TRAF3-mediated suppression of IL-17 signaling is responsible for its inhibitory regulation of EAE development.

DISCUSSION

Investigations from mouse models have shown that IL-17 plays critical roles in the pathogenesis of many inflammatory diseases, although the evidence for effective anti–IL-17 therapy in human autoimmune diseases is still lacking (Jäger et al., 2009). Although some recent studies have begun to uncover IL-17–mediated signal transduction, the negative regulation of IL-17 signaling still remains largely unknown (Shen et al., 2009; for review see Qian et al., 2010). In this study, we identify the first receptor proximal negative regulator TRAF3 in IL-17–mediated signaling associated with autoimmune diseases. TRAF3 was transiently recruited to IL-17R upon IL-17 stimulation. Binding of TRAF3 to IL-17R interfered with the formation of the activation complex IL-17R–Act1–TRAF6, resulting in suppressed IL-17–mediated signaling and suppressed induction of inflammatory genes. Moreover, TRAF3 also inhibited IL-17–induced expression of inflammatory cytokines and chemokines in vivo. Consistently, TRAF3 suppressed the development of both MOG-induced EAE and Th17 transfer–induced EAE. Collectively, our results suggest that TRAF3 functions as an IL-17R proximal regulator in critical control of IL-17–mediated inflammatory responses and autoimmune diseases like EAE.

IL-17 has been found to activate many signaling pathways for robust induction of proinflammatory cytokines and chemokines. Thus, negative regulation of IL-17 signaling is critical to shut off persistent inflammatory responses for the prevention of inflammation-mediated pathogenesis. So far, not much is known about how IL-17 signaling is negatively modulated (Shen et al., 2009). In this study, we found that IL-17 stimulation results in quick and transient recruitment of TRAF3 to IL-17R to timely turn off IL-17–mediated immediate signaling, including NF-κB and MAPKs. Consistently, we observed that TRAF3 dramatically suppressed IL-17–mediated induction of proinflammatory factors. It is noteworthy that IL-17 has been shown to synergize with TNF for amplification of inflammatory responses. Consistent with the repressive role of TRAF3 in IL-17 signaling, we found that TRAF3 also inhibited the synergistic induction of inflammatory genes by IL-17 and TNF. It has been reported that C/EBP-δ is responsible for the synergistic induction of IL-6 (Ruddy et al., 2004). Therefore, we checked whether TRAF3 affects the pathway of C/EBP in IL-17 signaling. We found that the induction of C/EBP-δ by IL-17 alone or by the combination of IL-17 and TNF was dramatically enhanced in TRAF3-deficient MEFs, whereas the induction was blocked in Act1-deficient MEFs (Fig. S5 A), which is consistent with the general inhibitory role of TRAF3 in IL-17 signaling. In addition to its role in the transcriptional induction of inflammatory genes, IL-17 can also stabilize messenger RNAs (mRNAs) of chemokines induced by TNF for persistent inflammatory responses. We found that knockdown of TRAF3 dramatically extended the half-life of the *KC* chemokine induced by TNF, whereas knockdown of Act1 greatly shortened the half-life of *KC* (Fig. S5, B–D), indicating that TRAF3 also plays an important inhibitory role in IL-17–mediated mRNA stability. Because TRAF6 is required for IL-17–mediated NF-κB and JNK activation (Schwandner et al., 2000) but not required for IL-17–mediated mRNA stability of *KC* (Hartupee et al., 2009), these results indicate that TRAF3 functions upstream of TRAF6 and likely at the IL-17R level for its general inhibitory effects in IL-17 signaling. Then we checked whether TNF affects the TRAF3–IL-17R interaction. We found that TNF did not affect the IL-17–induced interaction of TRAF3 with IL-17R (Fig. S5 E), suggesting that the synergy happens downstream of the IL-17R, likely through C/EBP or mRNA stability pathways. Because IL-17F also signals through IL-17RA and IL-17RC heterodimers like IL-17A, it is likely that TRAF3 may also repress IL-17F signaling. Indeed, we found that overexpression of TRAF3 inhibited whereas knockdown of TRAF3 enhanced the IL-17F–mediated signaling (Fig. S6). Interestingly, IL-17RA is also required for IL-17E (IL-25)–mediated function. It remains to be determined whether TRAF3 also plays a similar inhibitory role in IL-25–mediated signaling.

Deletion and point mutation analyses revealed that one TRAF3-binding site (PAEE) was responsible for IL-17R to associate with TRAF3. We further found that TRAF3 was recruited to IL-17R upon IL-17 stimulation, suggesting that TRAF3 really works at the receptor level. We recently characterized Act1 as an essential adaptor in IL-17–mediated signaling and inflammatory pathogenesis of autoimmune diseases (Qian et al., 2007). Because both TRAF3 and Act1 associate with IL-17R but function in the opposite way, we propose that TRAF3 may interfere with the binding of Act1 to IL-17R. Indeed, overexpression and knockdown experiments confirmed that TRAF3 competes with Act1 to interact with IL-17R. Moreover, TRAF3 also competes with TRAF6 to associate with IL-17R. Importantly, TRAF3 almost lost its inhibitory function when its binding domain to IL-17R was deleted, which is consistent with the competition mechanism for TRAF3 to function. Besides, when mapping the domains of IL-17R to interact with TRAF6, we unexpectedly found that TRAF6 interacts with the SEF domain instead of the TRAF-binding site in the distal domain used by TRAF3. As Act1 has been proved to interact with the SEF domain of IL-17R, these findings suggest that TRAF6 likely binds to IL-17R through Act1, which has TRAF6-binding sites (Qian et al., 2007). Our data that TRAF3 and Act1 bind to different domains of IL-17R and compete with each other to associate with the receptor suggest that binding of TRAF3 to IL-17R may lead to the conformational change of IL-17R and thus interfere with its association with Act1, resulting in the subsequent suppression of downstream signaling and induction of inflammatory genes.

TRAF3 has been found to be a critical regulator in both innate and adaptive immunity. It is an essential signaling adaptor in type I IFN producing signaling pathways for antiviral immunity, while it also controls noncanonical NF-κB activation through degrading NIK in CD40 and BAFF-mediated signaling in regulation of B cell functions. In contrast to its negative role in CD40 signaling, TRAF3 plays a positive role in LMP1-mediated signaling and functions, suggesting that TRAF3 may use different mechanisms to function in different signaling pathways. In this study, we found TRAF3 functions in a new signaling pathway with a different signaling mechanism. Although CD40 and BAFF lead to TRAF3 degradation through the cIAP1/2 signaling complex to turn on signaling, TRAF3 is not degraded upon IL-17 stimulation (Fig. S7). In addition, we demonstrate that TRAF3 competes with Act1 or TRAF6 to bind to IL-17R to turn off signaling for the prevention of continuous activation of inflammatory responses. Further dissection of mechanisms for TRAF3-mediated signaling would help us understand how TRAF3 exerts diverse roles in physiological and pathological conditions.

IL-17 has been shown to be critical for pathogenesis of many autoimmune diseases in mouse models. EAE is the well-recognized mouse model with symptoms and pathology resembling MS. Because TRAF3 plays a critical role in the regulation of IL-17–mediated inflammatory responses, we wondered whether TRAF3 is important in regulating IL-17–mediated pathology of EAE. For that purpose, we generated TRAF3 transgenic mice. Consistent with the negative role of TRAF3 in IL-17 signaling in cell culture system, TRAF3 inhibited

IL-17–mediated induction of inflammatory mediators in vivo in TRAF3 transgenic mice. Consistently, TRAF3 delayed the onset of MOG-induced EAE and also dramatically suppressed the disease incidence and severity of EAE, likely caused by inhibition of the production of inflammatory factors like cytokines, chemokines, and MMPs in CNS tissues. We also used a lentiviral system to knock down TRAF3 in mice and found that silencing of TRAF3 significantly increased IL-17–mediated induction of proinflammatory genes, including cytokines, chemokines, and MMPs in CNS tissues, and EAE clinical pathology, which is consistent with the data from the TRAF3 transgenic mice. Thus, our experiments from both transgenic and knockdown mice have demonstrated the critical control role of TRAF3 in EAE development. Further research is still necessary to uncover the importance of TRAF3 in the control of other autoimmune diseases such as CIA and inflammatory bowel disease. It also remains to be determined whether there are polymorphisms in TRAF3 that may account for some cases of autoimmunity.

It has been shown that TRAF3 is essential for TLR-mediated production of IL-10 and type I IFN, which are important suppressors of EAE development. TRAF3 is supposed to mediate the production of IL-10 and type I IFN because of the adjuvant effect to activate TLR signaling during EAE induction. Interestingly, we unexpectedly observed no significant change of IL-10 and only slight increase of type I IFN in the CNS of TRAF3 transgenic mice during EAE induction. One explanation could be that the endogenous TRAF3 expression level in the CNS may be high enough to mediate TLR signaling so that the increased TRAF3 expression in the transgenic mice only had a mild effect in the promotion of type I IFN and IL-10 production. Another possibility of no difference of IL-10 production could be that TRAF3 is essential for TLR-mediated IL-10 induction pathway while it inhibits BAFF-mediated IL-10 production in B cells, both of which may be involved during EAE induction. These results suggest that IL-10 and type I IFN may not be responsible for the suppressed EAE in the transgenic mice. Indeed, our results from antibody blockage support the conclusion that IL-10 and type I IFN are not important for TRAF3-mediated suppression of EAE development. We then performed Th17 cell transfer to assess the inhibitory effect of TRAF3 on EAE and indeed found that TRAF3 dramatically suppressed EAE induced by Th17 cell transfer, suggesting that TRAF3-mediated suppression of IL-17 signaling is responsible for its inhibitory regulation of EAE development.

In summary, we provide evidence for a previously unknown key regulatory mechanism by discovering that TRAF3 is critical in negative regulation of IL-17–mediated inflammatory responses and pathogenesis of EAE. TRAF3 functions through its binding to IL-17R to interfere with the formation of the activation signaling complex IL-17R–Act1–TRAF6. To our knowledge, TRAF3 represents the first receptor proximal negative regulator in IL-17 signaling. TRAF3 may serve as a potential new target for intervention of inflammatory autoimmune diseases like MS.

MATERIALS AND METHODS

Mice. To generate TRAF3 transgenic mice, cDNA encoding mouse TRAF3 was cloned into the plasmid pCAGGS (provided by J. Miyazaki, Osaka University, Suita, Osaka, Japan). Transgene-positive mice were identified by PCR using primers derived from the transgene construct and TRAF3 cDNA. The primer sequences used are as follows: sense primer 5′-GAAGGAAATGGGCGGGGAG-3′ on the pCAGGS promoter and antisense primer 5′-CGGCACTTCTCGCACTTGTA-3′ on the mouse TRAF3 cDNA. The mice were initially created on the CBA × C57BL/6 background by Shanghai Biomodel Organism Science & Technology Development Co., Ltd. and then backcrossed to C57BL/6 for six generations. The TRAF3 transgenic mice and littermate controls at 6–12 wk of age were used for experiments. C57BL/6 mice were purchased from Shanghai Laboratory Animal Center, Chinese Academy of Sciences. All mice were maintained in pathogen-free conditions. All animal experiments were performed in compliance with the guide for the care and use of laboratory animals and were approved by the institutional biomedical research ethics committee of the Shanghai Institutes for Biological Sciences (Chinese Academy of Sciences).

Reagents, cell lines, and constructs. Recombinant IL-17A, IL-17F, and TNF were purchased from R&D Systems. Anti-Flag (M2) antibody was purchased from Sigma-Aldrich. Anti-HA antibody was purchased from Covance. Antibodies to p-IκBα, p-p65, p-p38, p-JNK, ERK1/2, and C/EBP-δ were purchased from Cell Signaling Technology. Antibodies to p-ERK, IκBα, TRAF3, Act1, actin, IL-17RA, and Myc were purchased from Santa Cruz Biotechnology, Inc. Actinomycin D was purchased from Sigma-Aldrich. IFNAR1 and IL-10 neutralization antibody and relative isotype IgG were purchased from eBioscience.

HEK293 cells, HeLa cells, human astrocytes U87-MG, WT and TRAF3-deficient MEFs (provided by G. Cheng, University of California, Los Angeles, Los Angeles, CA; He et al., 2006), human primary FLSs (provided by N. Li, Shanghai Jiao Tong University School of Medicine, Shanghai, China; Zhang et al., 2009), and mouse primary astrocytes, purified as described previously (Qian et al., 2007), were maintained in DME supplemented with 10% (vol/vol) FBS (Hyclone), 100 μg/ml penicillin G, and 100 μg/ml streptomycin. HA- or M2 (Flag)-tagged TRAF3 or M2 (Flag)-tagged IL-17R and their deletion mutants were generated by PCR and then cloned into pcDNA3.1.

Lentivirus-delivered siRNA gene knockdown. The two siRNA sequences for human TRAF3 gene knockdown are 5′-GAAGGTTTCCTTGTTG-CAGAATGAA-3′ and 5′-AGAGTCAGGTTCCGATGAT-3′. The two siRNA sequences for human Act1 gene knockdown are 5′-GCTTCAGAA-CACTCATGTCTA-3′ and 5′-GCTGAAACCAATCCCAGAATA-3′. The siRNA sequence for mouse TRAF3 gene is as described previously (Matsuzawa et al., 2008) and for mouse Act1 is 5′-GTCAATTGCT-GAAACCGAT-3′. The scrambled control siRNA sequence is 5′-GGAT-CCTTGACAATACCAA-3′. The siRNA sequences were cloned into pLSLG lentiviral vector. The respective lentiviral vectors and help vectors were transfected to 293FT cells for viral packaging. 60 h after transfection, virus was collected to infect target cells in the presence of 10 mg/ml polybrene (Sigma-Aldrich). After 4 d, the cells were used in the experiments as described in Figs. 1 (B and D–F), 2 (B, D, and E), and 4 (D and E) and Figs. S1 (C–F), S5 (B–D), and S6.

Retrovirus-mediated gene expression. Flag-tagged TRAF3 was cloned into pMSCV-IRES-GFP vector. For restoration of gene expression, TRAF3-deficient MEFs were infected by viral supernatant suspensions obtained from 293FT cells transfected with Flag-tagged TRAF3 plasmid together with help vectors.

Coimmunoprecipitation. HEK293 cells were transfected with plasmids using the calcium phosphate transfection method, whereas HeLa cells were transfected with plasmids using Lipofectamine 2000 (Invitrogen) according

to the manufacturer's instructions. Cells were treated with or without IL-17 at different time points. Cell extracts were incubated with 0.5 µg individual antibody and 20 µl protein A beads (GE Healthcare). After overnight incubation, beads were washed four times with lysis buffer, separated by SDS-PAGE, and analyzed by immunoblotting.

Immunoblot analysis. Cells or mouse tissues were directly lysed in Triton buffer (20 mM Hepes and 0.5% Triton X-100, pH 7.6) and separated by 10% SDS-PAGE. Immunoblot analysis was performed by initial transfer of proteins onto polyvinylidene fluoride filters using Mini Trans-Blot (Bio-Rad Laboratories) and followed by a blocking step using Tris-buffered saline with 0.1% Tween 20 plus 5% nonfat dried milk for 1 h at room temperature. The filters were then incubated with primary antibody overnight and subsequently washed. The blots were then incubated with a secondary antibody conjugated with HRP for 1 h at room temperature. After extensive washing of the blots, signals were visualized with chemiluminescent HRP substrate (Millipore). Sometimes, the same blot was stripped off and reprobed with other antibodies to check their proteins.

Real-time RT-PCR. Total RNA was extracted from cells and mouse tissues with TRIZOL reagent (Invitrogen) according to the manufacturer's instructions. The cDNA was synthesized with PrimeScript RT reagent kit (Takara Bio Inc.). The expression of the genes encoding *KC*, *IL-6*, *CCL20*, *CXCL2*, *MMP3*, *IL-17*, *IL-10*, *IFNα4*, and *IFNβ* was quantified by real-time PCR with SYBR Premix ExTaq kit (Takara Bio Inc.). All gene expression results were normalized to the expression of housekeeping gene *Rpl13a*. Amplification of cDNA was performed on an AbiPrism 7900 HT cycler (Applied Biosystems). To calculate mRNA $t_{1/2}$, the mRNA level for the gene of interest was normalized to the *Rpl13a* mRNA level and plotted as log of the percentage of remaining mRNA versus time. The best fit to linear decay was determined, and the $t_{1/2}$ was calculated from the intersection at the point corresponding to 50% residual RNA.

Luciferase reporter assay. HeLa cells or Traf3$^{-/-}$ MEFs were cotransfected with 250 ng of an NF-κB–luciferase reporter and 25 ng of pRL-TK Renilla construct in each well of 12-well plates. After 24 h, cells were treated with 50 ng/ml IL-17 for 6 h before collection. Luciferase activity was measured with the Dual-Luciferase Reporter Assay system (Promega).

Adenoviral-mediated cytokine production in mice. mIL-17 was cloned into pAdTrack-CMV and then recombined with pAdEasy-1. The recombinant plasmid Ad-mIL-17 or empty vector (Ad-EV) was transfected to 293A cells, and recombinant adenoviral plaques were isolated and further purified by two rounds of plaque assays as described previously (He et al., 1998). After titration and dilution to a final concentration of 10^{10}–10^{11} virus per ml PBS, the adenovirus containing empty vector or mIL-17 was injected i.v. into WT or TRAF3 transgenic mice. mIL-17 expression was evaluated 4 d after injection.

ELISA. The cytokine and chemokine production from the sera of IL-17–treated mice was assessed with IL-6, KC, and IL-17 ELISA kits (R&D Systems) according to the manufacturer's instructions. A standard curve was generated using known amounts of the respective purified recombinant mouse cytokines or chemokines.

Induction and evaluation of EAE. The encephalitogenic peptide of MOG used to induce EAE corresponded to residues (Met-Glu-Val-Gly-Trp-Tyr-Arg-Ser-Pro-Phe-Ser-Arg-Val-Val-His-Leu-Tyr-Arg-Asn-Gly-Lys) 35–55. The peptide was purchased from China Peptides Biotechnology. Acute EAE was induced by a subcutaneous immunization with 300 µg of the MOG (35–55) peptide in CFA containing 5 mg/ml heat-killed H37Ra strain of *Mycobacterium tuberculosis* (Difco) in the back region. Pertussis toxin (List Biological Laboratories, Inc.) at a dose of 200 µg/mouse in PBS was administered i.v. on the day of immunization and once more 48 h after. Mice were weighed and examined daily for disease symptoms. They were scored for disease severity using the EAE scoring scale: 0, no clinical signs; 1, limp tail; 2, paraparesis (weakness, incomplete paralysis of one or two hind limbs); 3, paraplegia (complete paralysis of two hind limbs); 4, paraplegia with fore limb weakness or paralysis; and 5, moribund state or death.

Histology. Tissues for histological analysis were removed from mice 16 d after immunization and immediately fixed in 4% paraformaldehyde. Paraffin-embedded 5–10-µm sections of spinal cord were stained with H&E or luxol fast blue and then examined by light microscopy.

Bone marrow chimeras. Recipient WT control mice or TRAF3 transgenic mice were lethally irradiated by 800 cGy before injection in the tail vein of 5×10^6 mixed bone marrow cells from donor WT mice. Irradiated, transplanted mice were fed with drinking water containing 2 mg/ml neomycin sulfate (BioShop Canada Inc.) for 2 wk. After 8 wk of bone marrow reconstitution, EAE was induced as described in Induction and evaluation of EAE.

Adoptive transfer. Mice were immunized with MOG (35–55) plus complete Freund's adjuvant in conditions that induce active EAE. LNs were collected 10 d later, and single-cell suspensions were prepared. Cells (6×10^6 cells/ml) were cultured in RPMI 1640 medium (supplemented with 10% [vol/vol] FBS, 2 mM L-Gln, 1 mM sodium pyruvate, 100 IU/ml penicillin-streptomycin, and 20 mM 2-mercaptoethanol) with 20 mg/ml MOG (35–55) and 20 ng/ml IL-23 for 4 d for Th17 cell differentiation. The Th17 cells were then injected i.v. into recipient mice (2×10^7 cells per mouse) that had been sublethally irradiated (500 rads) at 4 h before injection as described previously (Kang et al., 2010).

Intracerebroventricular injection of lentiviruses. Preparation of lentivirus was described in Lentivirus-delivered siRNA gene knockdown. Viral supernatant was filtered through a 0.45-µm filter and concentrated by ultracentrifugation at 130,000 *g* for 2 h at 4°C and stored at −80°C. Before stereotaxic injection, the animals were anesthetized by intraperitoneal injection of 4% chloral hydrate and placed in a stereotaxic frame. Injection coordinates relative to bregma were as follows: AP, −0.22 mm; ML, −1.15 mm; and DV, −2.06 mm. A stainless steel cannula (5-µl Hamilton syringe) was inserted, and one deposit of 2 µl of lentivirus was slowly injected over a 2-min period. 5 min passed before the needle was removed to minimize retrograde flow along the needle track. After 4 d of intracerebroventricular injection, EAE was induced as described in Induction and evaluation of EAE.

Statistics. Differences in the expression of genes between the groups were analyzed by the Mann-Whitney *U* test. Two-tailed Student's *t* test was used to analyze the differences between the groups. One-way analysis of variance was initially performed to determine whether an overall statistically significant change existed before using the two-tailed paired or unpaired Student's *t* test. A p-value of <0.05 was considered statistically significant.

Online supplemental material. Fig. S1 shows that TRAF3 represses IL-17–induced NF-κB activation and downstream gene induction. Fig. S2 shows that TRAF3 interacts with IL-17RA and IL-17RC. Fig. S3 shows that TRAF3 binds to a TRAF-binding site in IL-17R. Fig. S4 shows that TRAF6 binds to the SEFIR domain of IL-17R and competes with TRAF3 to associate with IL-17R. Fig. S5 shows that TRAF3 suppresses IL-17–mediated induction of C/EBP-δ expression and stabilization of KC mRNA. Fig. S6 shows that TRAF3 represses IL-17F–induced signaling. Fig. S7 shows that IL-17 does not regulate TRAF3 expression. Online supplemental material is available at http://www.jem.org/cgi/content/full/jem.20100703/DC1.

We thank Dr. Weidong Le (Institute of Health Sciences, Shanghai Institutes for Biological Sciences, Chinese Academy of Sciences and Shanghai Jiao Tong University School of Medicine, Shanghai, China) for support in the delivery of lentivirus into the CNS of mice.

This work is supported by grants from the National Natural Science Foundation of China (nos. 30930084, 30871298, and 30928025), 973 program (no. 2010CB529705), Chinese Academy of Sciences (nos. KSCX2-YW-R-146 and KSCX1-YW-22), and Science and Technology Commission of Shanghai Municipality (nos. 08PJ14110, 09ZR1437100, and 10JC1416600).

The authors have no conflicting financial interests.

Submitted: 9 April 2010
Accepted: 19 October 2010

REFERENCES

Bettelli, E., T. Korn, and V.K. Kuchroo. 2007. Th17: the third member of the effector T cell trilogy. *Curr. Opin. Immunol.* 19:652–657. doi:10.1016/j.coi.2007.07.020

Chang, S.H., H. Park, and C. Dong. 2006. Act1 adaptor protein is an immediate and essential signaling component of interleukin-17 receptor. *J. Biol. Chem.* 281:35603–35607. doi:10.1074/jbc.C600256200

Cheng, G., A.M. Cleary, Z.S. Ye, D.I. Hong, S. Lederman, and D. Baltimore. 1995. Involvement of CRAF1, a relative of TRAF, in CD40 signaling. *Science.* 267:1494–1498. doi:10.1126/science.7533327

Chiang, E.Y., G.A. Kolumam, X. Yu, M. Francesco, S. Ivelja, I. Peng, P. Gribling, J. Shu, W.P. Lee, C.J. Refino, et al. 2009. Targeted depletion of lymphotoxin-alpha-expressing TH1 and TH17 cells inhibits autoimmune disease. *Nat. Med.* 15:766–773. doi:10.1038/nm.1984

Chung, J.Y., Y.C. Park, H. Ye, and H. Wu. 2002. All TRAFs are not created equal: common and distinct molecular mechanisms of TRAF-mediated signal transduction. *J. Cell Sci.* 115:679–688.

Cua, D.J., B. Hutchins, D.M. LaFace, S.A. Stohlman, and R.L. Coffman. 2001. Central nervous system expression of IL-10 inhibits autoimmune encephalomyelitis. *J. Immunol.* 166:602–608.

Fillatreau, S., C.H. Sweenie, M.J. McGeachy, D. Gray, and S.M. Anderton. 2002. B cells regulate autoimmunity by provision of IL-10. *Nat. Immunol.* 3:944–950. doi:10.1038/ni833

Gaffen, S.L. 2008. An overview of IL-17 function and signaling. *Cytokine.* 43:402–407. doi:10.1016/j.cyto.2008.07.017

Gaffen, S.L. 2009. Structure and signalling in the IL-17 receptor family. *Nat. Rev. Immunol.* 9:556–567. doi:10.1038/nri2586

Gardam, S., F. Sierro, A. Basten, F. Mackay, and R. Brink. 2008. TRAF2 and TRAF3 signal adapters act cooperatively to control the maturation and survival signals delivered to B cells by the BAFF receptor. *Immunity.* 28:391–401.

Gonzalez-García, I., Y. Zhao, S. Ju, Q. Gu, L. Liu, J.K. Kolls, and B. Lu. 2009. IL-17 signaling-independent central nervous system autoimmunity is negatively regulated by TGF-beta. *J. Immunol.* 182:2665–2671.

Häcker, H., V. Redecke, B. Blagoev, I. Kratchmarova, L.C. Hsu, G.G. Wang, M.P. Kamps, E. Raz, H. Wagner, G. Häcker, et al. 2006. Specificity in Toll-like receptor signalling through distinct effector functions of TRAF3 and TRAF6. *Nature.* 439:204–207. doi:10.1038/nature04369

Hartupee, J., C. Liu, M. Novotny, D. Sun, X. Li, and T.A. Hamilton. 2009. IL-17 signaling for mRNA stabilization does not require TNF receptor-associated factor 6. *J. Immunol.* 182:1660–1666.

He, J.Q., B. Zarnegar, G. Oganesyan, S.K. Saha, S. Yamazaki, S.E. Doyle, P.W. Dempsey, and G. Cheng. 2006. Rescue of TRAF3-null mice by p100 NF-κB deficiency. *J. Exp. Med.* 203:2413–2418. doi:10.1084/jem.20061166

He, J.Q., S.K. Saha, J.R. Kang, B. Zarnegar, and G. Cheng. 2007. Specificity of TRAF3 in its negative regulation of the noncanonical NF-kappa B pathway. *J. Biol. Chem.* 282:3688–3694.

He, T.C., S. Zhou, L.T. da Costa, J. Yu, K.W. Kinzler, and B. Vogelstein. 1998. A simplified system for generating recombinant adenoviruses. *Proc. Natl. Acad. Sci. USA.* 95:2509–2514.

Huang, F., C.Y. Kao, S. Wachi, P. Thai, J. Ryu, and R. Wu. 2007. Requirement for both JAK-mediated PI3K signaling and ACT1/TRAF6/TAK1-dependent NF-kappaB activation by IL-17A in enhancing cytokine expression in human airway epithelial cells. *J. Immunol.* 179:6504–6513.

Hunter, C.A. 2007. Act1-ivating IL-17 inflammation. *Nat. Immunol.* 8:232–234. doi:10.1038/ni0307-232

Ivanov, I.I., L. Zhou, and D.R. Littman. 2007. Transcriptional regulation of Th17 cell differentiation. *Semin. Immunol.* 19:409–417. doi:10.1016/j.smim.2007.10.011

Jabara, H., D. Laouini, E. Tsitsikov, E. Mizoguchi, A. Bhan, E. Castigli, F. Dedeoglu, V. Pivniouk, S. Brodeur, and R. Geha. 2002. The binding site for TRAF2 and TRAF3 but not for TRAF6 is essential for CD40-mediated immunoglobulin class switching. *Immunity.* 17:265–276.

Jäger, A., V. Dardalhon, R.A. Sobel, E. Bettelli, and V.K. Kuchroo. 2009. Th1, Th17, and Th9 effector cells induce experimental autoimmune encephalomyelitis with different pathological phenotypes. *J. Immunol.* 183:7169–7177.

Kang, Z., C.Z. Altuntas, M.F. Gulen, C. Liu, N. Giltiay, H. Qin, L. Liu, W. Qian, R.M. Ransohoff, C. Bergmann, et al. 2010. Astrocyte-restricted ablation of interleukin-17-induced Act1-mediated signaling ameliorates autoimmune encephalomyelitis. *Immunity.* 32:414–425. doi:10.1016/j.immuni.2010.03.004

Kolls, J.K., and A. Lindén. 2004. Interleukin-17 family members and inflammation. *Immunity.* 21:467–476. doi:10.1016/j.immuni.2004.08.018

Komiyama, Y., S. Nakae, T. Matsuki, A. Nambu, H. Ishigame, S. Kakuta, K. Sudo, and Y. Iwakura. 2006. IL-17 plays an important role in the development of experimental autoimmune encephalomyelitis. *J. Immunol.* 177:566–573.

Korn, T., E. Bettelli, M. Oukka, and V.K. Kuchroo. 2009. IL-17 and Th17 Cells. *Annu. Rev. Immunol.* 27:485–517.

Laan, M., J. Lötvall, K.F. Chung, and A. Lindén. 2001. IL-17-induced cytokine release in human bronchial epithelial cells in vitro: role of mitogen-activated protein (MAP) kinases. *Br. J. Pharmacol.* 133:200–206.

Lindén, A. 2007. A role for the cytoplasmic adaptor protein Act1 in mediating IL-17 signaling. *Sci. STKE.* 2007:re4 doi:10.1126/stke.3982007re4

Liu, C., W. Qian, Y. Qian, N.V. Giltiay, Y. Lu, S. Swaidani, S. Misra, L. Deng, Z.J. Chen, and X. Li. 2009. Act1, a U-box E3 ubiquitin ligase for IL-17 signaling. *Sci. Signal.* 2:ra63. doi:10.1126/scisignal.2000382

Lock, C., G. Hermans, R. Pedotti, A. Brendolan, E. Schadt, H. Garren, A. Langer-Gould, S. Strober, B. Cannella, J. Allard, et al. 2002. Gene-microarray analysis of multiple sclerosis lesions yields new targets validated in autoimmune encephalomyelitis. *Nat. Med.* 8:500–508. doi:10.1038/nm0502-500

Maitra, A., F. Shen, W. Hanel, K. Mossman, J. Tocker, D. Swart, and S.L. Gaffen. 2007. Distinct functional motifs within the IL-17 receptor regulate signal transduction and target gene expression. *Proc. Natl. Acad. Sci. USA.* 104:7506–7511.

Matsuzawa, A., P.H. Tseng, S. Vallabhapurapu, J.L. Luo, W. Zhang, H. Wang, D.A. Vignali, E. Gallagher, and M. Karin. 2008. Essential cytoplasmic translocation of a cytokine receptor-assembled signaling complex. *Science.* 321:663–668.

McGeachy, M.J., and D.J. Cua. 2008. Th17 cell differentiation: the long and winding road. *Immunity.* 28:445–453. doi:10.1016/j.immuni.2008.03.001

Moseley, T.A., D.R. Haudenschild, L. Rose, and A.H. Reddi. 2003. Interleukin-17 family and IL-17 receptors. *Cytokine Growth Factor Rev.* 14:155–174. doi:10.1016/S1359-6101(03)00002-9

Nakae, S., A. Nambu, K. Sudo, and Y. Iwakura. 2003. Suppression of immune induction of collagen-induced arthritis in IL-17-deficient mice. *J. Immunol.* 171:6173–6177.

Novatchkova, M., A. Leibbrandt, J. Werzowa, A. Neubüser, and F. Eisenhaber. 2003. The STIR-domain superfamily in signal transduction, development and immunity. *Trends Biochem. Sci.* 28:226–229.

O'Shea, J.J., S.M. Steward-Tharp, A. Laurence, W.T. Watford, L. Wei, A.S. Adamson, and S. Fan. 2009. Signal transduction and Th17 cell differentiation. *Microbes Infect.* 11:599–611.

Oganesyan, G., S.K. Saha, B. Guo, J.Q. He, A. Shahangian, B. Zarnegar, A. Perry, and G. Cheng. 2006. Critical role of TRAF3 in the Toll-like receptor-dependent and -independent antiviral response. *Nature.* 439:208–211. doi:10.1038/nature04374

Ogura, H., M. Murakami, Y. Okuyama, M. Tsuruoka, C. Kitabayashi, M. Kanamoto, M. Nishihara, Y. Iwakura, and T. Hirano. 2008. Interleukin-17 promotes autoimmunity by triggering a positive-feedback

loop via interleukin-6 induction. *Immunity*. 29:628–636. doi:10.1016/j.immuni.2008.07.018

Ouyang, W., J.K. Kolls, and Y. Zheng. 2008. The biological functions of T helper 17 cell effector cytokines in inflammation. *Immunity*. 28:454–467. doi:10.1016/j.immuni.2008.03.004

Park, H., Z. Li, X.O. Yang, S.H. Chang, R. Nurieva, Y.H. Wang, Y. Wang, L. Hood, Z. Zhu, Q. Tian, and C. Dong. 2005. A distinct lineage of CD4 T cells regulates tissue inflammation by producing interleukin 17. *Nat. Immunol.* 6:1133–1141.

Pineda, G., C.K. Ea, and Z.J. Chen. 2007. Ubiquitination and TRAF signaling. *Adv. Exp. Med. Biol.* 597:80–92.

Prinz, M., H. Schmidt, A. Mildner, K.P. Knobeloch, U.K. Hanisch, J. Raasch, D. Merkler, C. Detje, I. Gutcher, J. Mages, et al. 2008. Distinct and nonredundant in vivo functions of IFNAR on myeloid cells limit autoimmunity in the central nervous system. *Immunity*. 28:675–686. doi:10.1016/j.immuni.2008.03.011

Qian, Y., C. Liu, J. Hartupee, C.Z. Altuntas, M.F. Gulen, D. Jane-Wit, J. Xiao, Y. Lu, N. Giltiay, J. Liu, et al. 2007. The adaptor Act1 is required for interleukin 17-dependent signaling associated with autoimmune and inflammatory disease. *Nat. Immunol.* 8:247–256. doi:10.1038/ni1439

Qian, Y., Z. Kang, C. Liu, and X. Li. 2010. IL-17 signaling in host defense and inflammatory diseases. *Cell. Mol. Immunol.* 7:328–333. doi:10.1038/cmi.2010.27

Reboldi, A., C. Coisne, D. Baumjohann, F. Benvenuto, D. Bottinelli, S. Lira, A. Uccelli, A. Lanzavecchia, B. Engelhardt, and F. Sallusto. 2009. C-C chemokine receptor 6-regulated entry of TH-17 cells into the CNS through the choroid plexus is required for the initiation of EAE. *Nat. Immunol.* 10:514–523. doi:10.1038/ni.1716

Regev, L., E. Ezrielev, E. Gershon, S. Gil, and A. Chen. 2010. Genetic approach for intracerebroventricular delivery. *Proc. Natl. Acad. Sci. USA*. 107:4424–4429.

Ruddy, M.J., G.C. Wong, X.K. Liu, H. Yamamoto, S. Kasayama, K.L. Kirkwood, and S.L. Gaffen. 2004. Functional cooperation between interleukin-17 and tumor necrosis factor-alpha is mediated by CCAAT/enhancer-binding protein family members. *J. Biol. Chem.* 279:2559–2567.

Saijo, K., B. Winner, C.T. Carson, J.G. Collier, L. Boyer, M.G. Rosenfeld, F.H. Gage, and C.K. Glass. 2009. A Nurr1/CoREST pathway in microglia and astrocytes protects dopaminergic neurons from inflammation-induced death. *Cell*. 137:47–59. doi:10.1016/j.cell.2009.01.038

Sato, K., A. Suematsu, K. Okamoto, A. Yamaguchi, Y. Morishita, Y. Kadono, S. Tanaka, T. Kodama, S. Akira, Y. Iwakura, et al. 2006. Th17 functions as an osteoclastogenic helper T cell subset that links T cell activation and bone destruction. *J. Exp. Med.* 203:2673–2682. doi:10.1084/jem.20061775

Schwandner, R., K. Yamaguchi, and Z. Cao. 2000. Requirement of tumor necrosis factor receptor-associated factor (TRAF)6 in interleukin 17 signal transduction. *J. Exp. Med.* 191:1233–1240. doi:10.1084/jem.191.7.1233

Sebkova, L., A. Pellicanò, G. Monteleone, B. Grazioli, G. Guarnieri, M. Imeneo, F. Pallone, and F. Luzza. 2004. Extracellular signal-regulated protein kinase mediates interleukin 17 (IL-17)-induced IL-8 secretion in *Helicobacter pylori*-infected human gastric epithelial cells. *Infect. Immun.* 72:5019–5026.

Shen, F., M.J. Ruddy, P. Plamondon, and S.L. Gaffen. 2005. Cytokines link osteoblasts and inflammation: microarray analysis of interleukin-17- and TNF-alpha-induced genes in bone cells. *J. Leukoc. Biol.* 77:388–399.

Shen, F., N. Li, P. Gade, D.V. Kalvakolanu, T. Weibley, B. Doble, J.R. Woodgett, T.D. Wood, and S.L. Gaffen. 2009. IL-17 receptor signaling inhibits C/EBPbeta by sequential phosphorylation of the regulatory 2 domain. *Sci. Signal.* 2:ra8. doi:10.1126/scisignal.2000066

Stromnes, I.M., and J.M. Goverman. 2006. Active induction of experimental allergic encephalomyelitis. *Nat. Protoc.* 1:1810–1819. doi:10.1038/nprot.2006.285

Toy, D., D. Kugler, M. Wolfson, T. Vanden Bos, J. Gurgel, J. Derry, J. Tocker, and J. Peschon. 2006. Cutting edge: interleukin 17 signals through a heteromeric receptor complex. *J. Immunol.* 177:36–39.

Tseng, P.H., A. Matsuzawa, W. Zhang, T. Mino, D.A. Vignali, and M. Karin. 2010. Different modes of ubiquitination of the adaptor TRAF3 selectively activate the expression of type I interferons and proinflammatory cytokines. *Nat. Immunol.* 11:70–75.

Vallabhapurapu, S., A. Matsuzawa, W. Zhang, P.H. Tseng, J.J. Keats, H. Wang, D.A. Vignali, P.L. Bergsagel, and M. Karin. 2008. Nonredundant and complementary functions of TRAF2 and TRAF3 in a ubiquitination cascade that activates NIK-dependent alternative NF-kappaB signaling. *Nat. Immunol.* 9:1364–1370.

VanArsdale, T.L., S.L. VanArsdale, W.R. Force, B.N. Walter, G. Mosialos, E. Kieff, J.C. Reed, and C.F. Ware. 1997. Lymphotoxin-beta receptor signaling complex: role of tumor necrosis factor receptor-associated factor 3 recruitment in cell death and activation of nuclear factor kappaB. *Proc. Natl. Acad. Sci. USA*. 94:2460–2465. doi:10.1073/pnas.94.6.2460

Xie, P., B.S. Hostager, and G.A. Bishop. 2004. Requirement for TRAF3 in signaling by LMP1 but not CD40 in B lymphocytes. *J. Exp. Med.* 199:661–671. doi:10.1084/jem.20031255

Xie, P., L.L. Stunz, K.D. Larison, B. Yang, and G.A. Bishop. 2007. Tumor necrosis factor receptor-associated factor 3 is a critical regulator of B cell homeostasis in secondary lymphoid organs. *Immunity*. 27:253–267. doi:10.1016/j.immuni.2007.07.012

Ye, P., F.H. Rodriguez, S. Kanaly, K.L. Stocking, J. Schurr, P. Schwarzenberger, P. Oliver, W. Huang, P. Zhang, J. Zhang, et al. 2001. Requirement of interleukin 17 receptor signaling for lung CXC chemokine and granulocyte colony-stimulating factor expression, neutrophil recruitment, and host defense. *J. Exp. Med.* 194:519–527.

Zarnegar, B.J., Y. Wang, D.J. Mahoney, P.W. Dempsey, H.H. Cheung, J. He, T. Shiba, X. Yang, W.C. Yeh, T.W. Mak, et al. 2008. Noncanonical NF-kappaB activation requires coordinated assembly of a regulatory complex of the adaptors cIAP1, cIAP2, TRAF2 and TRAF3 and the kinase NIK. *Nat. Immunol.* 9:1371–1378.

Zhang, Q., J. Wu, Q. Cao, L. Xiao, L. Wang, D. He, G. Ouyang, J. Lin, B. Shen, Y. Shi, et al. 2009. A critical role of Cyr61 in interleukin-17-dependent proliferation of fibroblast-like synoviocytes in rheumatoid arthritis. *Arthritis Rheum.* 60:3602–3612. doi:10.1002/art.24999