

## **Requirement of Tumor Necrosis Factor Receptor-associated Factor (TRAF)6 in Interleukin 17 Signal Transduction**

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### **Abstract**

Signaling through its widely distributed cell surface receptor, interleukin (IL)-17 enhances the transcription of genes encoding proinflammatory molecules. Although it has been well documented that IL-17 activates the transcription factor nuclear factor (NF)- $\kappa$ B and c-Jun NH<sub>2</sub>-terminal kinase (JNK), the upstream signaling events are largely unknown. Here we report the requirement of tumor necrosis factor receptor-associated factor (TRAF)6 in IL-17-induced NF- $\kappa$ B and JNK activation. In embryonic fibroblasts (EFs) derived from TRAF6 knockout mice, IL-17 failed to activate the I $\kappa$ B kinases (IKKs) and JNK. Consequently, IL-17-induced IL-6 and intercellular adhesion molecule 1 expression in the TRAF6-deficient cells was abolished. Lack of TRAF6 appeared to be the sole defect responsible for the observed failure to respond to IL-17, because transient transfection of TRAF6 expression plasmid into the TRAF6-deficient cells restored IL-17-induced NF- $\kappa$ B activation in a luciferase reporter assay. Furthermore, the levels of IL-17 receptor (IL-17R) on the TRAF6-deficient EFs were comparable to those on the wild-type control cells. Defect in IL-17 response was not observed in TRAF2-deficient EFs. Moreover, when TRAF6 and IL-17R were coexpressed in 293 cells, TRAF6 coimmunoprecipitated with IL-17R. Together, these results indicate that TRAF6, but not TRAF2, is a crucial component in the IL-17 signaling pathway leading to proinflammatory responses.

Key words: cytokines • inflammation • signaling • kinases • transcription

### **Introduction**

IL-17, previously termed CTLA-8 (cytotoxic T cell lymphocyte-associated antigen 8; reference 1), is a 20–30-kD protein secreted by activated CD4 cells, mainly Th0 and Th1 cells (2, 3). In different cell types, IL-17 induces the expression of a large variety of proinflammatory molecules, including IL-1 $\beta$ , IL-8, inducible nitric oxide synthase, cyclooxygenase-2, and intercellular adhesion molecule (ICAM)-1 (3–6). IL-17 also reportedly induces hematopoietic cytokines such as GM-CSF, leukemia inhibitory factor, and IL-6 (7). Thus, IL-17 appears to provide a direct linkage between T cell activation and inflammatory responses. Indeed, IL-17 has been implicated in Th1-mediated inflammatory diseases such as rheumatoid arthritis and organ transplant rejections (7–11).

Like TNF and IL-1, which elicit similar cellular responses, IL-17 also activates the transcription factors nuclear factor (NF)- $\kappa$ B and activator protein (AP)-1, impor-

tant mediators of gene regulatory activities exhibited by proinflammatory cytokines (6, 12–14). AP-1 is activated by a phosphorylation event mediated by mitogen-activated protein kinases, including c-Jun NH<sub>2</sub>-terminal kinase (JNK)-1 (15), whereas NF- $\kappa$ B activation is preceded by the activation of I $\kappa$ B kinase- $\alpha$  and - $\beta$  (IKKs), which are present in most cell types as a heterocomplex (16–21). The IKKs phosphorylate two specific serine residues on the I $\kappa$ B proteins, marking them for proteolysis (22–26). The degradation of the inhibitory I $\kappa$ B proteins results in the release and nuclear translocation of the NF- $\kappa$ B proteins, which transcriptionally activate target genes.

TNF and IL-1 signal through different cell surface receptors and utilize different sets of receptor-proximal signaling molecules. TNF induces the trimerization of its type I receptor (the major signaling receptor relative to the type II receptor), which initiates a signaling cascade engaging the adapter TRADD (TNF receptor-associated death domain protein), the serine/threonine kinase RIP (receptor-interacting protein), and TNF receptor-associated factor (TRAF)2 (27–29). IL-1 induces the heterocomplex forma-

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tion of two different receptor chains, the type I receptor (IL-1R1) and the receptor accessory protein (IL-1RAcP) (30–32). This triggers the signaling events that involve the adapter molecule MyD88, the serine/threonine kinase IRAK (IL-1 receptor-associated kinase), and TRAF6 (33–35). TRAF2 and TRAF6 mediate the activation of both IKK and JNK (36). Therefore, the TRAF proteins represent a class of adapters that lead distinct upstream signaling pathways for different cytokines to a point of convergence.

The receptor of IL-17 (IL-17R) is a type 1 transmembrane protein with an apparent molecular mass of 130 kD (37). It does not share sequence homology with the receptors of TNF, IL-1, or other cytokines. Little is known about the molecular signaling mechanism of IL-17R. In this study, we used TRAF2- and TRAF6-deficient mouse embryonic fibroblasts (MEFs) to study the role of TRAF molecules in IL-17-induced NF- $\kappa$ B and JNK activation. Our results indicate that TRAF6 is a critical signaling molecule in the IL-17 signaling pathway.

## Materials and Methods

**Cell Culture and Biological Material.** Primary EFs were gifts from T.W. Mak's laboratory (Amgen Institute/Ontario Cancer Institute, Toronto, Ontario, Canada). The cells were derived from embryos at day 14.5 of gestation as described previously (38, 39). EFs and human embryonic kidney (HEK) 293 cells were cultured in high glucose DME (Cellgro) supplemented with 10% certified FBS, 10 mM glutamine, and 50  $\mu$ g/ml each of streptomycin and penicillin (GIBCO BRL) in a humidified incubator with 5% CO<sub>2</sub>. Mouse TNF was provided by Genentech; human IL-1 $\beta$  and mouse IL-17 were purchased from Biosource.

**Luciferase Reporter Assays.** To reconstitute IL-17 response, TRAF6<sup>+/-</sup> and TRAF6<sup>-/-</sup> MEFs were seeded in six-well (35 mm) plates and transfected on the following day with 0.5  $\mu$ g of luciferase reporter gene controlled by a basic promoter and six NF- $\kappa$ B binding sites, 2  $\mu$ g of pRSV- $\beta$ -gal, and 50 ng of either vector DNA or TRAF6 expression plasmid DNA (34) using the Effectene Transfection Reagent (QIAGEN Inc.) according to manufacturer-recommended protocol. 36 h after transfection, the cells were stimulated with TNF (25 g/ml) or IL-17 (100 g/ml) for 8 h. Luciferase activity and  $\beta$ -galactosidase activity was determined with the Luciferase Assay System (Promega Corp.) and chemiluminescent reagents from Tropix, respectively.

**Electrophoretic Mobility Assay.** To assay NF- $\kappa$ B-DNA binding activity, 2  $\times$  10<sup>5</sup> EFs were seeded in 3.5-cm dishes for 24 h. The cells were cultured in serum-free medium for 1 h before the addition of cytokines. At indicated times (see Fig. 1) after cytokine induction, the cells were harvested with PBS containing 1 mM EDTA. Nuclear extract preparation and gel electrophoretic mobility shift assay (EMSA) were performed as described previously (40).

**In Vitro Kinase Assay and Immunoblotting Analysis.** EFs (10<sup>6</sup>) were either left untreated or were treated with the indicated cytokines (see Fig. 2) for 10 min in the absence of serum. Cells were rinsed with cold PBS and lysed on ice for 20 min in lysis buffer (50 mM Hepes, pH 7.6; 125 mM NaCl; 1.5 mM MgCl<sub>2</sub>; 10% glycerol; 0.5% NP-40; 1 mM EGTA; protease inhibitor cocktail [Boehringer Mannheim]; 20 mM  $\beta$ -glycerophosphate; 1 mM sodium orthovanadate; and 5 mM *p*-nitro-phenylphos-

phate). Cell debris was pelleted by centrifugation at 14,000 rpm for 20 min. IKK-NEMO (NF- $\kappa$ B essential modulator) protein complex in the supernatants was immunoprecipitated using anti-NEMO rabbit antiserum and assayed for activity that phosphorylated recombinant I $\kappa$ B- $\alpha$  (amino acids 1–250) as previously described (40). JNK was precipitated with anti-JNK-1 mAb (PharMingen), and the kinase activity was detected in an in vitro kinase assay using recombinant GST-c-Jun 1-79 fusion protein as a substrate (Santa Cruz Biotechnology).

To detect IKK- $\alpha$  and IKK- $\beta$  immunoprecipitated with anti-NEMO antiserum, the immunoprecipitates were separated by 10% SDS-PAGE, transferred to nitrocellulose membrane, and immunoblotted with anti- $\alpha$ -IKK and anti- $\beta$ -IKK polyclonal antibodies (Santa Cruz Biotechnology) as described earlier (40). JNK-1 was detected with an anti-JNK-1 rabbit polyclonal antibody (Santa Cruz Biotechnology).

**Expression Plasmids, Transfection, and Coimmunoprecipitation.** Expression plasmids for Flag-tagged CD40 and Flag-tagged IL-1R1 were described elsewhere (33, 41). Mammalian expression plasmid for Flag-tagged IL-17R was constructed by inserting PCR-generated cDNA lacking the coding sequence for the signal peptide into expression vector pFlag-CMV-1 (Eastman Kodak Co.). For coprecipitation experiments, 2  $\times$  10<sup>6</sup> HEK 293 cells were plated on 10-cm dishes and transfected the following day with indicated amounts of expression constructs (see Fig. 5). After 18 h, the cells were collected, washed once with PBS, and lysed for 20 min on ice with lysis buffer. Cell lysates were incubated for 2 h with anti-Flag M2 beads (Sigma Chemical Co.). After extensive washing with lysis buffer, the beads were eluted with 400  $\mu$ M Flag peptide (Sigma Chemical Co.), separated by SDS-PAGE, and transferred to nitrocellulose membrane. Immunoblot analysis was performed with an anti-Myc or anti-HA (hemagglutinin) mAb (Babco). Expression of transfected constructs was verified by immunoblotting aliquot of cell lysates.

**Determination of IL-6 Secreted by MEFs.** EFs were seeded at a density of 3  $\times$  10<sup>4</sup> cells per well in 24-well plates and cultured for 24 h. Cells were left untreated or stimulated with TNF, IL-1, or IL-17 for 15 h. IL-6 concentration of the culture supernatant was determined by ELISA (Endogen) using manufacturer's protocols.

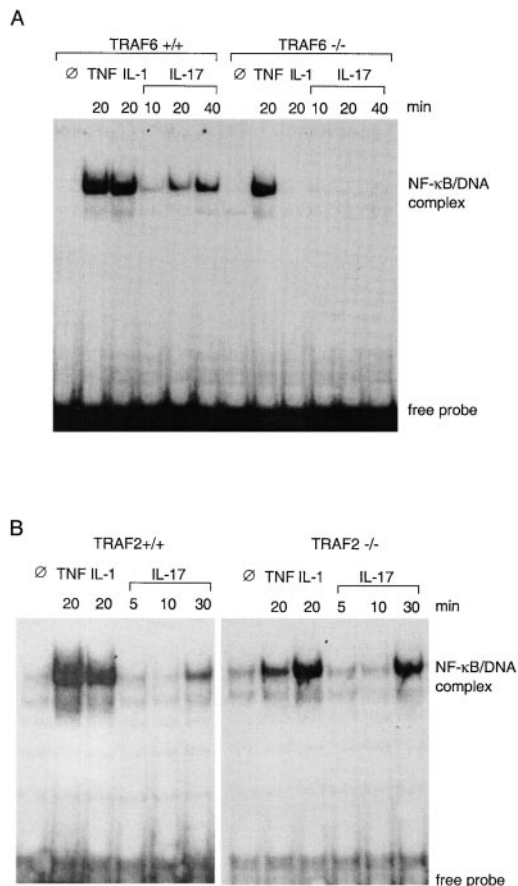
**Immunostaining of Cell Surface IL-17R and ICAM-1.** To detect IL-17R, EFs (5  $\times$  10<sup>5</sup>) were first incubated for 1 h with PBS containing goat anti-murine (m)IL-17R antiserum (Santa Cruz Biotechnology) or control serum. After washing twice with PBS, the cells were incubated for 1 h with FITC-conjugated antibodies to goat IgG (Santa Cruz Biotechnology). Flow cytometry was performed using a FACSCalibur™ Analyzer (Becton Dickinson). For detection of ICAM-1, cells were treated for 15 h with 10 ng/ml of TNF, IL-1, or IL-17 or were left untreated. The cells were first incubated with mAb to ICAM-1 (PharMingen) and then stained with FITC-conjugated antibodies to mouse IgG (Santa Cruz Biotechnology).

## Results and Discussion

**Impairment of IL-17-induced NF- $\kappa$ B Activation in TRAF6-deficient Cells.** EFs obtained from TRAF6<sup>-/-</sup> embryos or their wild-type littermates were stimulated with IL-17, TNF, or IL-1. NF- $\kappa$ B-DNA binding activity was examined by EMSA. In wild-type cells, NF- $\kappa$ B activation was detected 10 min after IL-17 treatment, followed by a continued increase up to 40 min (Fig. 1 A). Similar results

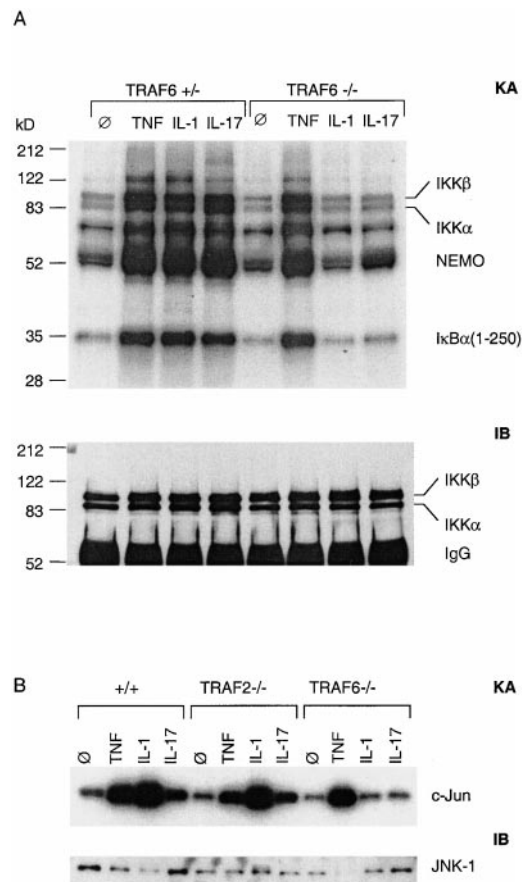
were obtained with heterozygous EF cells (TRAF6<sup>+/-</sup>; data not shown). However, in TRAF6<sup>-/-</sup> cells, no NF-κB activation was detected within the time frame examined. In agreement with a previous report, TRAF6<sup>-/-</sup> cells also failed to respond to IL-1 (38). In contrast, TNF-induced NF-κB activation was unaltered by TRAF6 gene disruption, indicating that TRAF6 deficiency only causes defect in response to specific cytokines.

Because TRAF2 has been also implicated in NF-κB activation by certain cytokines (39, 42–44), we examined if IL-17-induced NF-κB activation was affected by TRAF2 deficiency. Treatment of TRAF2<sup>-/-</sup> MEFs with either IL-17 or IL-1 induced NF-κB activity comparable to that observed in the wild-type control cells (Fig. 1 B), whereas NF-κB activation induced by 20 min of TNF treatment was significantly reduced. These results indicate that TRAF2 is involved in signal transduction of TNF, as described previously (39), but not in that of IL-1 or IL-17.



**Figure 1.** NF-κB activation in MEFs. (A) Lack of NF-κB activation in TRAF6-deficient cells in response to IL-1 and IL-17. Nuclear extracts were prepared from TRAF6<sup>+/-</sup> or TRAF6<sup>-/-</sup> MEFs treated for indicated length of time with 25 ng/ml mTNF, 50 ng/ml IL-1, or 100 ng/ml mIL-17. 6 μg of protein from each sample was tested for ability to bind to a synthetic DNA fragment containing an NF-κB binding site in an EMSA. (B) Normal NF-κB activation in TRAF2-deficient cells in response to IL-17. The same experiments as in A were performed with MEFs derived from TRAF2-deficient mouse embryos and their wild-type littermates.

**Abolishment of IL-17-induced IKK Activation in TRAF6-deficient Cells.** NF-κB activation induced by IL-1, TNF, and LPS entails the activation of IKKs. We investigated whether IL-17-induced NF-κB activation is also mediated by the IKKs, and if so, whether IL-17-induced IKK activation is impaired in the TRAF6 knockout cells. IKK complex in TRAF6<sup>+/-</sup> or TRAF6<sup>-/-</sup> EFs untreated or treated with TNF, IL-1, or IL-17 was coimmunoprecipitated with antibodies against NEMO, a stable component in the IKK complex (17, 19). Immunoblot analysis indicated that similar amounts of IKK-α and -β were immunoprecipitated from untreated or cytokine-treated TRAF6<sup>+/-</sup> and TRAF6<sup>-/-</sup> cells (Fig. 2). The immunoprecipitates were then assayed for activity to phosphorylate recombinant IκB-α (amino acids 1–250) containing IKK substrate, serines 32 and 36.

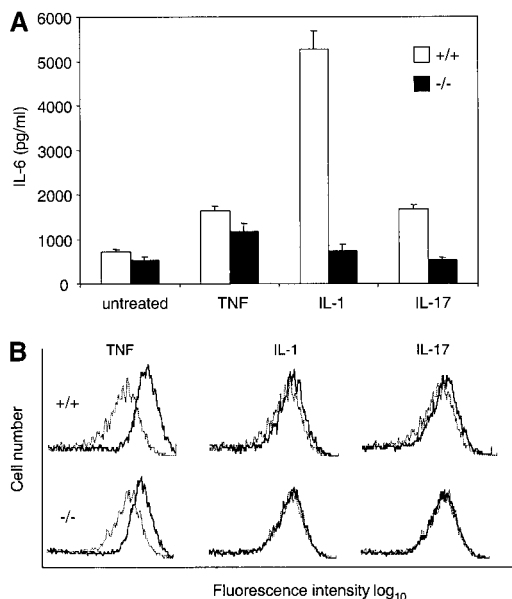


**Figure 2.** Kinase activity of the IKK complex and JNK isolated from TRAF6-deficient MEFs. (A) TRAF6-deficient (–/–) and heterozygous EFs (+/–) were treated with 25 ng/ml mTNF, 50 ng/ml IL-1, or 100 ng/ml mIL-17 for 10 min. The IKK complex was immunoprecipitated using anti-NEMO polyclonal antibodies and assayed for activity to phosphorylate IκB-α (amino acids 1–250) (top panel [KA], in vitro kinase assay). Immunoprecipitated materials were separated by SDS-PAGE and immunoblotted with a mixture of antibodies to IKK-α and IKK-β (bottom panel [IB], immunoblot). Molecular mass standards are shown in kilodaltons. (B) JNK-1 was precipitated from wild-type, TRAF2-deficient, and TRAF6-deficient MEFs treated with indicated cytokines and assayed for their activity to phosphorylate recombinant c-Jun (top panel). The amounts of precipitated JNK-1 were determined by immunoblotting (bottom panel).

Treatment of TRAF6<sup>+/-</sup> cells with TNF, IL-1, or IL-17 significantly enhanced the IKK activity, as evidenced by increased phosphorylation of I $\kappa$ B- $\alpha$ , NEMO, and IKKs (Fig. 2 A). On the other hand, IKK activity in the TRAF6<sup>-/-</sup> cells was enhanced only by TNF and not by IL-1 or IL-17. These results indicate that, like TNF and IL-1, IL-17 activates NF- $\kappa$ B by inducing the catalytic activity of the IKKs, and that IL-17-induced IKK activation involves TRAF6.

Similar results were obtained when JNK activity was measured in TRAF-deficient cells (Fig. 2 B). IL-17- and IL-1-induced JNK activation was abolished in TRAF6 knockout cells but remained intact in TRAF2 knockout cells. In agreement with an earlier report (39), a reduction of TNF-induced JNK activation was observed in TRAF2-deficient cells.

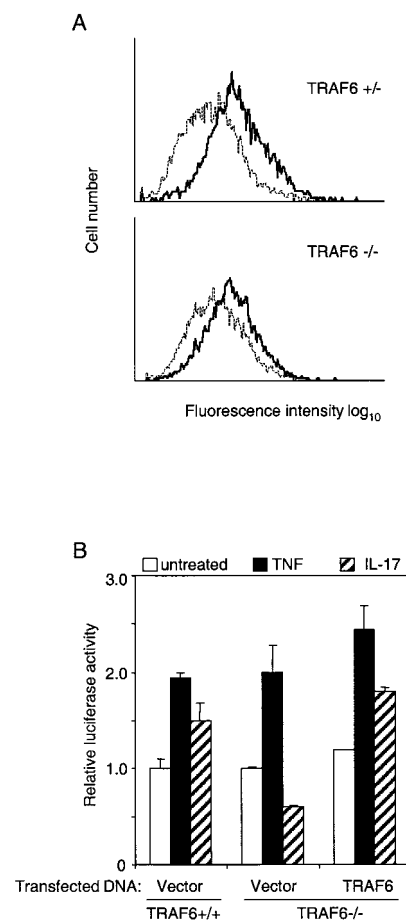
**Failure of IL-17 to Induce IL-6 and ICAM-1 Expression in TRAF6-deficient Cells.** To examine if the failure of IL-17 to activate IKK and NF- $\kappa$ B would be translated to its inability to activate target genes, we measured IL-6 secretion by TRAF6-deficient MEFs. Control MEFs responded to TNF, IL-1, and IL-17 with a significant increase in IL-6 secretion (Fig. 3 A). The TRAF6 knockout cells, on the other hand, failed to produce increased levels of IL-6 in response to either IL-17 or IL-1. An increase of IL-6 secretion by the TRAF6-deficient cells was observed after TNF treatment, albeit at a slightly lower level relative to that se-



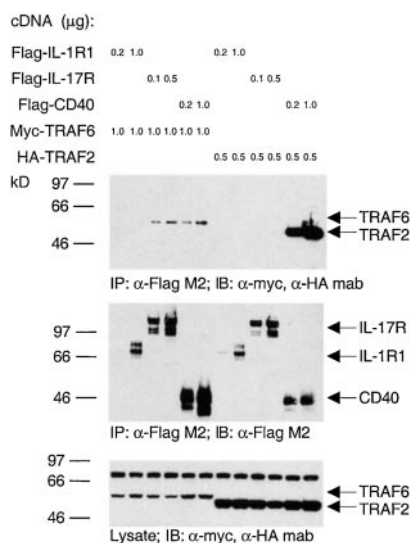
**Figure 3.** Target gene activation in TRAF6-deficient MEFs. (A) Failure of IL-17 to increase IL-6 production by the TRAF6-deficient EFs. Wild-type (+/+) or TRAF6-deficient (-/-) EFs were cultured in the presence of 10 ng/ml mTNF, 10 ng/ml IL-1, or 10 ng/ml mL-17 for 15 h. IL-6 in cell culture medium was determined by ELISA. Data shown are average values of three independent experiments  $\pm$  SD. (B) Failure of IL-17 to increase ICAM-1 expression on TRAF6-deficient cells. Wild-type or TRAF6<sup>-/-</sup> cells were treated with cytokines as in A. Levels of ICAM-1 protein on cell surface were determined by FACS<sup>®</sup> analysis using mouse mAbs specific to ICAM-1. Dotted line, untreated cells; solid line, cytokine-treated cells.

creted by control cells. Because TNF induces the release of IL-1, which could contribute to the net increase of IL-6 secretion by TNF-treated cells, this slight reduction in TNF response may be due to a defect in IL-1 response observed in the TRAF6<sup>-/-</sup> cells.

Similar results were obtained when ICAM-1 cell surface expression was measured by flow cytometry. All three cytokines induced ICAM-1 expression on wild-type MEFs, with the strongest induction observed after TNF treatment (Fig. 3 B). Although TNF-induced increase in ICAM-1 expression remained intact in TRAF6<sup>-/-</sup> cells, the effects of IL-1 and IL-17 were diminished. These results further support that IL-17-induced response gene activation is mediated by NF- $\kappa$ B and that TRAF-6 is indispensable for the gene regulatory activities of IL-17.



**Figure 4.** Surface Expression of IL-17R on MEFs and restoration of IL-17 response by transient introduction of TRAF6 protein into TRAF6<sup>-/-</sup> cells. (A) Determination of IL-17R on TRAF6<sup>+/+</sup> and TRAF6<sup>-/-</sup> MEFs by FACS<sup>®</sup> analysis using polyclonal antibody to mouse IL-17R. Dotted line, mouse-matched isotype IgG; solid line, anti-IL-17R. (B) Reconstitution of IL-17 response. Wild-type or TRAF6-deficient EFs were transfected with RSV- $\beta$ -gal vector, an NF- $\kappa$ B-dependent luciferase reporter construct, together with 50 ng of vector DNA or TRAF6 expression plasmid. Cells were left untreated (open bars) or stimulated with 25 ng/ml TNF (closed bars) or 100 ng/ml IL-17 (hatched bars) for 8 h. Luciferase activity was determined and normalized based on  $\beta$ -gal activity.



**Figure 5.** Interaction of TRAF6 and IL-17R. 293 cells were transfected with indicated amounts of expression plasmid DNA of Flag-tagged IL-1R1, IL-17R, or CD40 together with Myc-tagged TRAF6 or HA-tagged TRAF2 for 18 h. The cells were lysed, and the receptors were immunoprecipitated with anti-Flag beads. Top panel, the Flag immunoprecipitates were resolved by SDS-PAGE and blotted with antibodies to Myc and HA to detect coprecipitating TRAF6 and TRAF2. Center panel, the Flag immunoprecipitates were blotted with Flag antibody to check the amounts of precipitated receptors. Bottom panel, lysates of transfected cells were blotted with both Myc and HA antibodies to determine the expression levels of the TRAF2 and TRAF6 proteins.

**Determinative Effect of TRAF6 Deficiency in IL-17 Response.** To exclude the possibility that the observed unresponsiveness to IL-17 in TRAF6<sup>-/-</sup> cells might be due to insufficient surface expression of IL-17R, EFs were immunostained with an antiserum against mouse IL-17R and analyzed by flow cytometry. The results indicate that TRAF6<sup>+/-</sup> and TRAF6<sup>-/-</sup> cells expressed similar levels of IL-17R (Fig. 4 A).

To further eliminate the possibility that failure of TRAF6<sup>-/-</sup> cells to respond to IL-17 could be caused by defects other than TRAF6 deficiency, we tested if IL-17-induced NF- $\kappa$ B activation could be restored by introducing exogenous TRAF6 into these cells. TRAF6<sup>-/-</sup> cells were transiently transfected with an NF- $\kappa$ B-driven luciferase report construct together with a TRAF6 expression plasmid or a control vector. Mirroring NF- $\kappa$ B activity measured by EMSA (Fig. 1), IL-17 failed to induce luciferase activity in TRAF6<sup>-/-</sup> cells, although response to TNF appeared normal (Fig 4 B). TRAF6<sup>-/-</sup> cells transfected with TRAF6 expression plasmid responded to IL-17 with an increase of luciferase activity similar to that observed in control cells.

**Binding of TRAF6 to IL-17R.** Previous studies indicate that TRAF6 can be recruited to signaling pathways in two modes. It can either bind directly to the receptor intracellular domain, as in the signaling of CD40 and receptor activator of NF- $\kappa$ B (38, 45–47), or be recruited into the pathways by adapter proteins, as in the signaling of IL-1, IL-18, and LPS (48, 49). To determine if TRAF6 interacts

with IL-17R, we coexpressed Flag-tagged IL-17R, IL-1R1, or CD40 together with Myc-tagged TRAF6 in 293 cells. In agreement with our earlier finding, TRAF6 did not coimmunoprecipitate with IL-1R1 (34). In contrast, TRAF6 coimmunoprecipitated with IL-17R or CD40 (Fig. 5). To determine if this IL-17R–TRAF6 interaction is specific to TRAF6, the same set of Flag-tagged receptors was also coexpressed with HA-tagged TRAF2. TRAF2 was found in the immunoprecipitates of CD40, in agreement with previous reports (47, 50), but was not detected in the IL-17R complex. These observations further support the involvement of TRAF6, but not TRAF2, in IL-17 signaling. Because similar amounts of TRAF6 were detected in IL-17R and CD40 complex, TRAF6 may bind to IL-17R directly, although we cannot rule out a remote possibility that this interaction might be mediated by an abundant adapter molecule. The nature of the TRAF6 and IL-17R interaction will be addressed in detail when good antibodies to IL-17R become available.

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