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TNFR-Associated Factors 2 and 5 Differentially Regulate the Instructive IL-6 Receptor Signaling Required for Th17 Development

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IL-17–producing CD4$^+$ T cells (Th17 cells) regulate host defense and immune pathogenesis, and IL-6 plays an important role for the differentiation of Th17 cells. We have previously identified that TNFR-associated factor (TRAF)5 binds to the signal-transducing receptor gp130 through the C-terminal TRAF domain and inhibits Th17 development mediated by IL-6. Although gp130 has TRAF-binding motifs that can be recognized by other TRAF family proteins, it is unclear how TRAFs regulate IL-6–driven Th17 differentiation in general. Using retrovirus-mediated gene complementation and gene silencing approaches, we found that not only TRAF5 but also TRAF2 restrained the IL-6R signaling, whereas TRAF1, TRAF3, TRAF4, and TRAF6 did not. *Traf2* silencing further promoted the ability of naive CD4$^+$ T cells from *Traf5*$^{-/-}$ mice to differentiate into Th17 cells. Notably, TRAF5 but not TRAF2 expressed in naive CD4$^+$ T cells was rapidly downregulated after TCR triggering, which indicates that TRAF5 specifically inhibits instructive IL-6 signals in the initial stage of Th17 development. Collectively, our results demonstrate a dedicated role for TRAF2 and TRAF5 in the process of IL-6–mediated Th17 development and a differential role for TCR signaling in regulation of TRAF2 and TRAF5. Therefore, both TRAF2 and TRAF5 work as important regulators of the IL-6R signaling needed for Th17 development. ***The Journal of Immunology*****, 2016, 196: 4082–4089.**

Interleukin-17–producing CD4$^+$ T cells (Th17 cells) play critical roles in host defense against specific bacterial and fungal pathogens and in the pathogenesis of immune-mediated diseases such as multiple sclerosis, arthritis, inflammatory bowel diseases, psoriasis, and asthma. Th17 cells differentiate from Ag-recognized naive CD4$^+$ T cells in response to IL-6 and TGF-β (1–4). IL-6 binds to a receptor complex consisting of the ligand-binding subunit IL-6R (CD126) and the signaling subunit gp130 (CD130) and subsequently induces the formation of an IL-6–IL-6R–gp130 complex that is clustered into a dimer structure (5). The dimerized receptor complex activates STAT3 that is essential for inducing the lineage-specific transcription factor retinoic acid–related orphan receptor γt that is required for Th17 cell development (1, 6–8).

The six members of TNFR-associated factors (TRAF1–TRAF6) are intracellular signaling proteins that mediate the link between receptor-proximal molecular events and intracellular signaling proteins. The C-terminal domain of TRAFs, which is composed of a coiled-coil/leucine zipper domain (TRAF-N) followed by a TRAF domain (TRAF-C), contributes to homo- or hetero-oligomerization of TRAF proteins and their recognition of a variety of cytoplasmic and receptor proteins (9–11). There is growing evidence that TRAFs control the differentiation of CD4$^+$ T cells (12–19).

We have shown that TRAF5 inhibits the differentiation of Th17 cells by antagonizing signaling via the receptor for IL-6 (18). An enhanced Th17 response is induced in *Traf5*$^{-/-}$ CD4$^+$ T cells, and Th17 cell–associated experimental autoimmune encephalomyelitis is more exaggerated in *Traf5*$^{-/-}$ mice than in wild-type mice. Notably, TRAF5 constitutively associates with gp130 via its TRAF-C domain and negatively controls the STAT3 signaling mediated by IL-6. The cytoplasmic tail of gp130 contains TRAF-binding motifs that can be recognized by TRAF family proteins (20–22). From these studies, we hypothesized that the TRAF-binding motifs in gp130 could be recognized by TRAFs other than TRAF5 and that the TRAF–gp130 binding would be critical for the IL-6–driven Th17 differentiation.

In this study, we found that TRAF2 shared the TRAF-binding motifs with TRAF5 and also suppressed the IL-6R signaling required for Th17 development. Interestingly, expression of TRAF2 and TRAF5 was differentially controlled by TCR signals in CD4$^+$ T cells; that is, TRAF5 was rapidly downregulated in naive CD4$^+$ T cells after anti-CD3 stimulation, whereas TRAF2 was stably expressed in activated CD4$^+$ T cells during the course of Th17 development. Thus, TRAF2 and TRAF5 inhibit the IL-6R signaling with different kinetics during Th17 development. We propose a new signaling mechanism of Th17 differentiation that is critically regulated by both TRAF2 and TRAF5.

Materials and Methods

Mice

Traf5$^{-/-}$ and OT-II mice on a C57BL/6 (B6) background were described previously (18). B6 mice were from Japan SLC. CD45.1$^+$ B6 mice have

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Abbreviations used in this article: B6, C57BL/6; BAF-gp130, IL-3–dependent BAF/B03 pro–B cell lines stably expressing mouse gp130; NIK, NF-κB–inducing kinase; shRNA, short hairpin RNA; shTRAF2, shRNA that targets TRAF2; shTRAF3, shRNA that targets TRAF3; shTRAF5, shRNA that targets TRAF5; TRAF, TNFR-associated factor.

been described previously (23). All mice were bred and maintained under specific pathogen-free conditions, and experiments were in compliance with protocols approved by the Institute for Animal Experimentation, Tohoku University Graduate School of Medicine.

Peptide, chemicals, Abs, and cytokines

OVA peptide (amino acids 323–339) was from Anaspec. Dynabeads protein G (100-04D) and anti-V5 (R960-25) were from Life Technologies. Anti-CD3ε (low endotoxin, azide free; 145-2C11; 100331), anti-CD28 (low endotoxin, azide free; 37.51; 102112), anti–IFN-γ (low endotoxin, azide free; XMG1.2; 505827), anti–IL-4 (low endotoxin, azide free; 11B11; 504115), anti–IL-12 (low endotoxin, azide free; C17.8; 505304), PE–allophycocyanin–anti-IL-17A (TC11-18H10.1; 506905 or 506916), and PE–anti-IL-21R (4A9; 131906) were from BioLegend. Anti–IL-6 (low endotoxin, azide free; MP5-20F3; 16-7061) was from Affymetrix. Pacific Blue–allophycocyanin–anti-CD4 (RM4-5; 558107 or 553051), biotin–anti-CD132 (TUGm2; 554470), and streptavidin-PE (554061) were from BD Biosciences. Anti–c-Myc (9B11, 2276), anti-STAT3 (9139), Ab to STAT3 phosphorylated at Tyr^{705} (9145), and Alexa Fluor 647–-anti-phosho-STAT3 (4324) were from Cell Signaling Technology. Anti–c-Myc (9E10; sc-40), anti-gp130 (M-20; sc-656), anti–TRAF1 (N-19; sc-874), anti-TRAF3 (M-20; sc-947), anti-TRAF4 (H-72; sc-10776), anti-TRAF5 (C-19; sc-6195), and anti-TRAF6 (H-274; sc-7221) were from Santa Cruz Biotechnology. Anti–β-actin (C4; MAB1501) was from Millipore. Anti-gp130 (HMβ1; D022-3) and anti-TRAF2 (592) were from MBL International. Allophycocyanin–anti-gp130 (125623; FAB4681A) and anti–TGF-β1, 2, and 3 (1D11; MAB1835) were from R&D Systems. Recombinant human IL-2 (200-02), human IL-6 (200-06), soluble human IL-6R (200-06R), human TGF-β1 (100-21C), murine IL-3 (213-13) and murine IL-21 (210-21) were from PeproTech. Anti-Flag (DYKDDDDK, 018-22381) was from Wako Pure Chemical.

Plasmids and transfection

c-Myc–gp130 vectors encoding wild-type or mutants of gp130 were previously described (18). cDNA encoding mouse *Traf5* (MR208836) was from OriGene Technologies. Based on the published cDNA sequences of mouse *Traf1* (NM_009421.3), *Traf2* (NM_009422.2), *Traf3* (NM_011632.3), *Traf4* (NM_009423.4), and *Traf6* (NM_009424.2), cDNA of the entire coding regions was obtained by RT-PCR methods. TRAF1–TRAF6 retroviral vectors were constructed by insertion of each TRAF-encoding cDNA into pMX-IRES-EGFP (24, 25). To prepare retrovirus-mediated expression of nontargeting short hairpin RNA (shRNA) or shRNA that targets TRAF2 (shTRAF2), shRNA that targets TRAF3 (shTRAF3), or shRNA that targets TRAF5 (shTRAF5), the target sequence was inserted into pSIREN-RetroQ vector (BD Biosciences): nontargeting shRNA (5′-CAACAAGATGAAGAGCACCAA-3′), shTRAF2 (5′- GCGATCTT-CATCAAAGCTATT-3′), shTRAF3 (5′-GCAAGAGAGAGATTCTGGC-3′), and shTRAF5 (5′- CGGTCTCTGAGAGAATTGA-3′). TRAF2-V5 and TRAF2-FLAG vectors were generated by insertion of TRAF2-encoding cDNA into a pcDNA3.1/V5-HisA vector (Life Technologies) and a p3XFLAG-CMV-10 vector (Sigma-Aldrich E4401), respectively. DNA-encoding TRAF2 mutant constructs were generated with a PrimeSTAR mutagenesis basal kit (Takara Bio) according to the manufacturer's instructions. The correct DNA sequence of the newly generated constructs was verified with a 3100 genetic analyzer and a BigDye terminator kit (Life Technologies). FuGENE 6 or FuGENE HD (Roche) or polyethyleneimine (Sigma-Aldrich 408727) were used for transient transfection of HEK293T cells, as well as Plat-E and Plat-A retroviral packaging cells (24, 25). Retrovirus was produced by transfection of retrovirus vectors into Plat-E or Plat-A cells. Virus-containing supernatants at days 2 and 3 were pooled and then were concentrated by centrifugation at 8000 × *g* at 4˚C for 16 h. Supernatants containing 5 μg/ml Polybrene were added to naive $CD4^+$ T cell cultures 12 h after initial activation. The cells were spun at 800 × *g* for 1 h at 32˚C and were further cultured for 8 h, and then virus-containing supernatant was removed from the cultures and replaced with fresh medium.

Cell lines and proliferation assay

IL-3–dependent BAF/B03 pro–B cell lines stably expressing mouse gp130 (BAF-gp130) were maintained in RPMI 1640 containing 7% FCS and 20% WEHI-231 culture supernatant. For cell proliferation assay, BAF-gp130 cell lines were washed extensively to remove WEHI supernatants and plated into triplicate wells in a 96-well U-bottom plate at 5×10^3 cells per wells. Cells were cultured with graded doses of IL-3 or IL-6–IL-6R for 72 h, followed by addition of 1:10 culture volume of 5 mg/ml MTT solution in PBS (Sigma-Aldrich M2128) and cultured for additional 4 h. The water-insoluble MTT formazan was solubilized with DMSO, and the intensity was measured calorimetrically at 570 nm.

Flow cytometry

Cells were incubated with Fc block (2.4G2) before staining with Abs for cell surface and intracellular Ags. For intracellular cytokine staining, cells were stimulated with 50 ng/ml PMA and 1 μg/ml ionomycin (EMD Biosciences) for 5 h in the presence of GolgiPlug (BD Biosciences). After staining of surface markers, cells were fixed and permeabilized using Cytofix/Cytoperm and Perm/Wash buffer from BD Biosciences according to the manufacturer's instructions. For phospho-STAT3 (Tyr^{705}) staining, cells were fixed and permeabilized using Phosflow fix buffer I and Phosflow perm buffer III from BD Biosciences according to the manufacturer's instructions. Data were acquired on a FACSCanto II (BD Biosciences) and analyzed with FlowJo software (Tree Star).

T cell culture

Naive ($CD44^{low}CD62L^{high}$) $CD4^+$ T cells were purified from spleens of $Traf5^{-/-}$ or wild-type age-matched B6 or OT-II mice with a naive $CD4^+$ T cell isolation kit II (130-093-227, Miltenyi Biotec) and an autoMACS Pro cell separator (Miltenyi Biotec). Cells were cultured in RPMI 1640 medium with penicillin, streptomycin, glutamine, 2-ME, and 7% FCS. Naive $CD4^+$ T cells from B6 mice were plated at a density of 2.5×10^5 cells/ml and stimulated with 1 μg/ml plate-bound anti-CD3 and 1 μg/ml soluble anti-CD28. Th17 differentiation was initiated by addition of IL-6–IL-6R (30 or 100 ng/ml) and TGF-β1 (0.1 ng/ml) in the presence of blocking Abs to IFN-γ (1 μg/ml) and IL-4 (1 μg/ml). Naive OT-II $CD4^+$ T cells (at a density of 2.5×10^5 cells/ml) were activated for 12 h with 0.2 μM OVA peptide (amino acids 323–339) and irradiated T cell–depleted wild-type splenic APCs from B6 mice (at a density of 5×10^6 cells/ml) in the presence of IL-2 (5 ng/ml), IL-6–IL-6R (30 ng/ml), TGF-β1 (0.1 ng/ml), and blocking Abs to IFN-γ (2 μg/ml), IL-4 (2 μg/ml), and IL-12 (2 μg/ml), and then transduced with retroviral shRNA vectors, followed 4 d later by restimulation for 5 h with PMA and ionomycin.

Adoptive cell transfer experiment

$CD45.2^+$ naive $CD4^+$ T cells from wild-type or $Traf5^{-/-}$ OT-II mice were cultured in vitro for the first 12 h with OVA peptide and splenic APCs from wild-type B6 mice in the presence of IL-2 (5 ng/ml) and blocking Abs to IFN-γ (2 μg/ml), IL-4 (2 μg/ml), IL-12 (2 μg/ml), IL-6 (2 ng/ml), and TGF-β (2 ng/ml), and then transduced with a retroviral vector containing GFP and shTRAF2, followed 8 h later by adoptive transfer into $CD45.1^+$ congenic B6 mice. Recipient mice were immediately immunized by s.c. injection at the base of the tail with 2 mg OVA protein in CFA (Difco) containing 400 μg *Mycobacterium tuberculosis* to induce the differentiation of the donor OT-II cells in vivo. Recall IFN-γ and IL-17A responses in GFP^- or GFP^+ ($shTRAF2^+$) $CD45.2^+$ donor cell populations in the draining lymph nodes were assessed 7 d after immunization.

Immunoprecipitation and immunoblot analysis

Cells were lysed for 30 min in ice-cold 1% Nonidet P-40 buffer (20 mM Tris-HCl [pH 7.5], 150 mM NaCl, 2 mM EDTA, 1% Nonidet P-40, 50 mM NaF, 1 mM Na_3VO_4, and 10 mM *N*-ethylmaleimide, containing protease inhibitor mixture [P8340; Sigma-Aldrich]). Insoluble material was removed by centrifugation at 15,000 × *g* for 10 min. Protein content was determined by bicinchoninic acid assay (Thermo Scientific). Proteins were immunoprecipitated from lysates overnight at 4˚C with primary Abs immobilized on Dynabeads protein G. After being washed extensively with ice-cold lysis buffer, beads were boiled for 5 min at 100˚C in 4× lithium dodecyl sulfate sample buffer (NP0007; Life Technologies). Eluted sample were further reduced for 10 min at 70˚C with 2-ME for immunoblot analysis. Samples were separated by SDS-PAGE, transferred onto polyvinylidene difluoride membranes (Immobilon-P; Millipore) and analyzed by immunoblot with the appropriate Abs. All blots were developed with Immobilon Western HRP substrate (Millipore).

Real-time RT-PCR

SYBR Premix Ex Taq (Takara Bio) and a 7500 real-time PCR system (Life Technologies) were used for quantitative RT-PCR. Total RNA was extracted with TRIzol (Life Technologies), and cDNA was then synthesized with SuperScript III reverse transcriptase and $oligo(dT)_{20}$ (Life Technologies). Each transcript was analyzed concurrently on the same plate with the gene encoding β-actin, and results are presented relative to the abundance of transcripts encoding β-actin. Primers were as follows: *Traf1* (forward, 5′-AGATGATGAGGATCGGATCTGT-3′, reverse, 5′-TTGAAGGAA-

CAGCCAACACC-3′); *Traf2* (forward, 5′-CTGTCTGTCCCAATGATGGA-3′, reverse, 5′-CATTCCTGCTCAGTGTGGTG-3′); *Traf3* (forward, 5′- GAACCTGCTGAAGGAGTGGA-3′, reverse, 5′-GACTCGTTGTTTCGGAGCAT-3′); *Traf4* (forward, 5′-CCGGCTTCGACTACAAGTTC-3′, reverse, 5′-TCAGGGCATTTGAAGACTCC-3′); *Traf5* (forward, 5′-CCGACACCGAGTACCAGTTTG-3′, reverse, 5′-CGGCACCGAGTTCAATTCTC-3′); *Traf6* (forward, 5′-GAGTTTGACCCACCTCTGGA-3′, reverse, 5′-TTCATTGTCAACTGGGCACT-3′); *Actb* (forward, 5′-CTGCCTGACGGCCAGG-3′, reverse, 5′-GGAAAAGAGCCTCAGGGCAT-3′).

Statistical analysis

Statistical significance was assessed with a Student *t* test with two-sided distributions.

Results

TRAF5 is rapidly downregulated after stimulation with anti-CD3 in naive CD4$^+$ T cells

To investigate expression profiles of six TRAF family molecules in CD4$^+$ T cells during T cell activation, naive CD4$^+$ T cells from wild-type B6 mice were stimulated with plate-coated anti-CD3 Ab and soluble anti-CD28 Ab, and mRNA and protein expression levels of TRAFs were determined by real-time RT-PCR and immunoblotting, respectively. Whereas mRNA levels of TRAF1, TRAF3, TRAF4, and TRAF6 were greatly increased after stimulation, TRAF2 mRNA was almost unchanged (Fig. 1A, 1C, Supplemental Fig. 1). In contrast, TRAF5 mRNA was decreased in response to anti-CD3 and anti-CD28 (Fig. 1A). In line with this, TRAF5 protein and mRNA were rapidly downregulated within 4 h after stimulation with anti-CD3 and anti-CD28 (Fig. 1B, 1C). This downregulation of TRAF5 was largely dependent on anti-CD3 and was not influenced much by the presence of anti-CD28 or IL-6 (Fig. 1C). Alternatively, levels of TRAF2 mRNA and protein were stably maintained after anti-CD3 and anti-CD28 (Fig. 1B, 1C). These results demonstrated that the expression pattern of TRAF5 was different from those of other TRAFs and that TRAF5 expression was negatively regulated by TCR signaling in the beginning of naive T cell activation.

TRAF5 acts as a negative regulator of Th17 development by antagonizing early IL-6R signaling

We have shown that TRAF5 constitutively associates with a cytoplasmic region in gp130 and that TRAF5 restrains IL-6–driven Th17 differentiation (18). To investigate more precisely the role of TRAF5 in Th17 differentiation, wild-type naive CD4$^+$ T cells were stimulated with anti-CD3 and anti-CD28, and the amount of IL-6R proteins, gp130 and IL-6R, was measured by flow cytometry. Purified naive CD4$^+$ T cells already had IL-6R and gp130 on the surface, and both proteins gradually decreased after stimulation with anti-CD3 and anti-CD28 (Fig. 2A), which indicates that naive CD4$^+$ T cells become insensitive to IL-6 after activation. To explore the physiological role of IL-6R downregulation, we added a complex of IL-6 and IL-6R (IL-6–IL-6R) into differentiating naive CD4$^+$ T cell cultures at different time points to check how the levels of IL-6Rs influence Th17 differentiation. As expected, Th17 differentiation could not be induced when IL-6–IL-6R had been added at 48 h after initial activation (Fig. 2B). Thus, the downregulated IL-6R and gp130 on activated CD4$^+$ T cells are insufficient to promote the development of Th17 cells.

The above results suggested that the molecular complex of TRAF5 and gp130 could only inhibit early IL-6R signaling. If this were correct, TRAF5 marginally expressed in already activated wild-type CD4$^+$ T cells would be unable to inhibit IL-6R signaling. To test this, naive CD4$^+$ T cells from wild-type or *Traf5*$^{-/-}$ mice were exposed to IL-6–IL-6R from the beginning (0 h) or from 36 h after initial stimulation with anti-CD3 and anti-CD28, and then cells were cultured an additional 3 d to assess the development of IL-17A–producing cells. The expression of gp130 and IL-6R in *Traf5*$^{-/-}$ CD4$^+$ T cells was comparable to that in wild-type CD4$^+$ T cells (18) (data not shown). When IL-6–IL-6R had been added at 0 h, the proportion of *Traf5*$^{-/-}$ CD4$^+$ T cells that expressed IL-17A was significantly higher than that of wild-type CD4$^+$ T cells (Fig. 2B, 2C). Importantly, when IL-6–IL-6R had been added at 36 h, we found no significant difference between wild-type and *Traf5*$^{-/-}$ CD4$^+$ T cells in their expression of IL-17A (Fig. 2B, 2C), which strongly supports the hypothesis that TRAF5 inhibits the initial instructive IL-6R signaling that is essential for Th17 development.

TRAF2 is also inhibitory to IL-6R signaling and limits Th17 development mediated by IL-6

We have previously shown that the C-terminal domain of TRAF5 recognizes TRAF-binding motifs in gp130, which includes the amino acid sequence ^{774}VFSRSESTQPLLDSEERPEDLQLVD^{798}

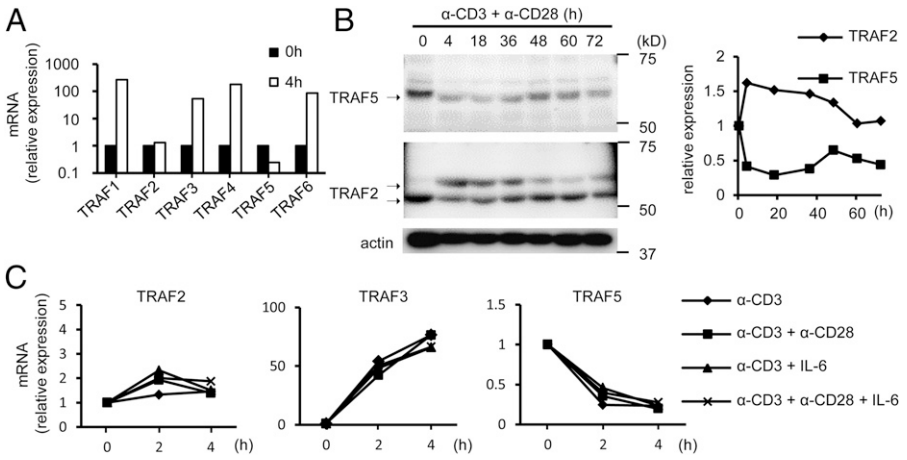

FIGURE 1. TRAF5 is downregulated in activated CD4$^+$ T cells, whereas TRAF1, TRAF2, TRAF3, TRAF4, and TRAF6 are not. (**A**) Quantitative RT-PCR analysis of TRAF family genes in activated CD4$^+$ T cells generated from wild-type naive CD4$^+$ T cells cultured for 4 h with anti-CD3 (1 μg/ml) and anti-CD28 (1 μg/ml), presented relative to the expression of the gene encoding β-actin. (**B**) Immunoblot analysis of TRAF2 and TRAF5 in wild-type naive CD4$^+$ T cells cultured for indicated time as in (A). *Right panel* shows densitometry ratio of TRAF2 or TRAF5 to β-actin. Arrows indicate the positions of TRAF2 and TRAF5. (**C**) Quantitative RT-PCR analysis of the expression of TRAF2, TRAF3, and TRAF5 genes in wild-type CD4$^+$ T cells cultured for indicated times with anti-CD3 (1 μg/ml) in the presence or absence of anti-CD28 (1 μg/ml) or IL-6 (10 ng/ml), presented relative to the expression of the gene encoding β-actin. Data are from one experiment representative of at least two independent experiments with similar results.

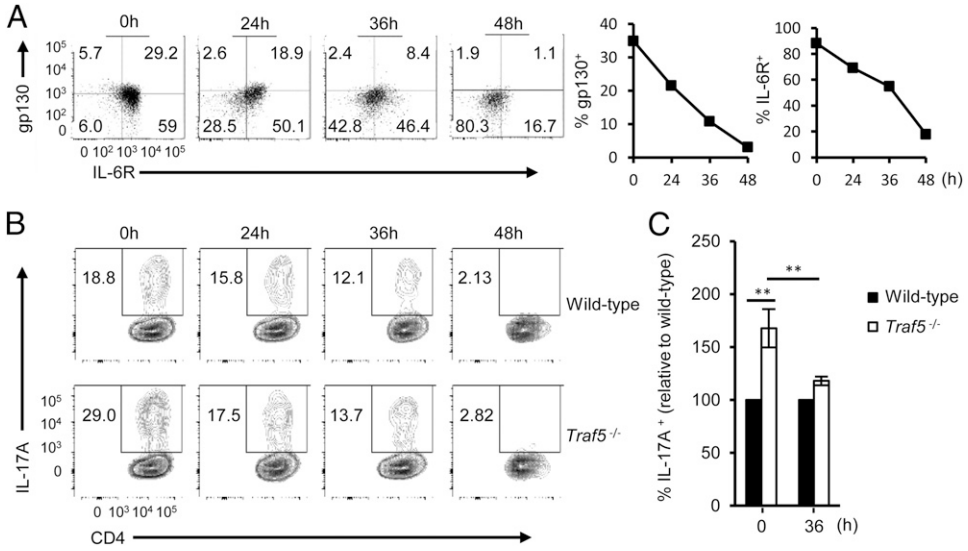

FIGURE 2. TRAF5 inhibits initial IL-6 signaling that is critical for Th17 differentiation. (**A**) Expression of gp130 and IL-6R on purified wild-type naive $CD4^+$ T cells cultured for indicated times with anti-CD3 and anti-CD28. Numbers in quadrants indicate the percentage of total $CD4^+$ T cells presented by the subset of interest. (**B**) Expression of IL-17A in activated $CD4^+$ T cells generated from wild-type or *Traf5*$^{-/-}$ naive $CD4^+$ T cells cultured with anti-CD3 and anti-CD28 in the presence of blocking Abs to IFN-γ (1 μg/ml) and IL-4 (1 μg/ml), followed by the induction of Th17 differentiation for an additional 3 d through the addition of IL-6–IL-6R (100 ng/ml) and TGF-β1 (0.1 ng/ml) at indicated times, then restimulation for 5 h with PMA and ionomycin. Numbers adjacent to outlined areas indicate percentage IL-$17A^+$ cells among total $CD4^+$ T cells. Data in (A) and (B) are from one experimental representative of at least two independent experiments with similar results. (**C**) Relative expression of IL-17A in activated $CD4^+$ T cells generated from wild-type or *Traf5*$^{-/-}$ naive $CD4^+$ T cells as in (B), followed by the induction of Th17 differentiation for an additional 3 d through the addition of IL-6–IL-6R (30 ng/ml) and TGF-β1 (0.1 ng/ml) at 0 or 36 h. Data in (C) are from three independent experiments (average and SD). $^{**}p < 0.01$ (Student *t* test).

containing an SXXE motif (^{776}SRSE^{779}) and two diacidic amino acids (^{788}EE^{789} and ^{792}ED^{793}) that have been identified as TRAF2-binding motifs (20–22). TRAF-C domains of TRAF1, TRAF2, TRAF3, and TRAF5 are thought to recognize the TRAF2-binding motifs (11). Thus, we hypothesized that other TRAFs also recognized the TRAF-binding motifs in gp130 and play important roles in gp130 signaling mediated by IL-6. To test this, BAF-gp130 cells, which stably express gp130 in an IL-3–dependent cell line BAF/B03, were further transduced with a vector encoding TRAF1, TRAF2, TRAF3, TRAF4, TRAF5, or TRAF6 to establish BAF-gp130 cell lines that stably overexpress respective TRAFs (Supplemental Fig. 2A). These cells had almost equivalent levels of GFP (Supplemental Fig. 2B) and cell surface gp130 (Supplemental Fig. 2C) and showed similar proliferative responses to IL-3 (Fig. 3A). Notably, the proliferative response induced by IL-6–IL-6R was significantly decreased in BAF-gp130 cells with TRAF2 or TRAF5 but not with TRAF1, TRAF3, or TRAF4 (Fig. 3B). Consistent with this, the phosphorylation of STAT3 mediated

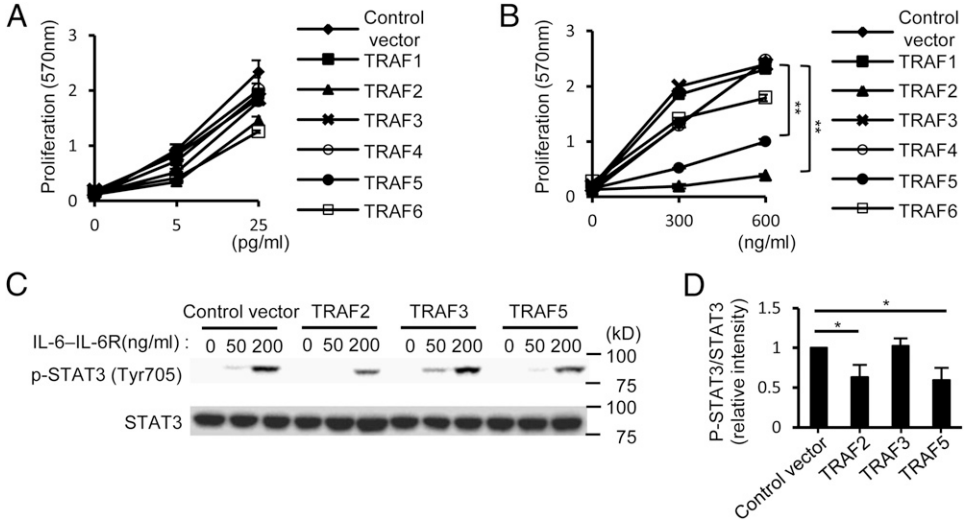

FIGURE 3. TRAF2 and TRAF5 inhibit IL-6–mediated proliferative responses of BAF cells. (**A** and **B**) Cell proliferative responses of BAF-gp130 cell lines stably transduced with retroviral vector expressing GFP alone (control vector) or GFP and TRAF1, TRAF2, TRAF3, TRAF4, TRAF5, or TRAF6, cultured for 3 d with indicated concentrations of IL-3 (A) or IL-6–IL-6R (B). Live cells at day 3 were evaluated with the colorimetric MTT assay. Data are mean values with SD of triplicate culture wells. Data are from one experiment representative of three independent experiments with similar results. $^{**}p < 0.01$ (Student *t* test). (**C**) Immunoblot analysis of STAT3 phosphorylated at Tyr^{705} (p-STAT3) and total STAT3 in BAF-gp130 cell lines as in (B) stimulated for 10 min with indicated concentrations of IL-6–IL-6R. (**D**) Ratio of phosphorylated STAT3 to total STAT3 in BAF-gp130 cell lines stimulated for 10 min with 200 ng/ml IL-6–IL-6R as in (C). Data are average with SEM of three independent experiments. $^{*}p < 0.05$ (Student *t* test).

by IL-6–IL-6R was impaired in BAF-gp130 cells with TRAF2 or TRAF5 but not with TRAF3 (Fig. 3C, 3D). BAF-gp130 with TRAF6 showed a marginal reduction in proliferation against IL-3 and IL-6 (Fig. 3A, 3B), which suggests that the overexpressed TRAF6 is inhibitory for the basal cellular activity in BAF/03 cells. These results show that both TRAF2 and TRAF5 have an ability to inhibit IL-6–mediated proliferation of BAF cells.

To examine whether the expression of TRAF2 is also inhibitory for Th17 development mediated by IL-6, we transduced differentiating wild-type naive CD4$^+$ T cells with a retroviral vector encoding GFP and TRAF1, TRAF2, or TRAF3, or TRAF4, TRAF5, or TRAF6 in polarizing conditions with IL-6, and assessed the frequency of IL-17A$^+$ cells in the GFP$^+$CD4$^+$ gated population. The expression of TRAF2 or TRAF5 significantly suppressed the development of IL-17A$^+$ T cells, but TRAF1, TRAF3, or TRAF4 did not (Fig. 4A, Supplemental Fig. 3). TRAF6 also inhibited the generation of IL-17A$^+$ T cells, suggesting a negative regulatory function of TRAF6 for TGF-β receptor signaling that is also required for Th17 development (15). We next transduced differentiating wild-type naive CD4$^+$ T cells with a retroviral vector encoding GFP and shRNA that targets mRNA encoding TRAF2 (shTRAF2), TRAF3 (shTRAF3), or TRAF5 (shTRAF5) and assessed the frequency of IL-17A$^+$GFP$^+$ CD4$^+$ cells. The efficiency of shRNA-mediated knockdown of each *Traf* gene was ~50–70% (data not shown). shTRAF2 upregulated the frequency of IL-17A$^+$ cells, whereas shTRAF3 and shTRAF5 could not (Fig. 4B). The reason why shTRAF5 did not significantly upregulate the IL-17$^+$ frequency may be that expression levels of TRAF5, gp130, and IL-6R had been greatly decreased at the time of transfection of shTRAF5, which resulted in cancelation of the activity of shTRAF5 in IL-6R signaling (see Figs. 1, 2). Collectively, these results clearly show that TRAF2 works as a negative regulator for IL-6–mediated Th17 differentiation and that TRAF2 can exhibit its inhibitory function even in the later phase of Th17 development.

Next, to confirm whether the overexpressed TRAF2 or TRAF5 is inhibitory for IL-6–regulated gp130 signaling in CD4$^+$ T cells, wild-type CD4$^+$ T cells with TRAF2 or TRAF5 were stimulated with IL-6–IL-6R to assess the phosphorylation of STAT3. The level of gp130 on CD4$^+$ T cells with TRAF2 or TRAF5 was comparable with that with control vector (Fig. 4C). Notably, TRAF2 or TRAF5 transduced CD4$^+$ T cells displayed significantly lower amounts of phosphorylated STAT3 than did control vector transduced CD4$^+$ T cells (Fig. 4D, 4E). The phosphorylation of STAT3 mediated by IL-21 was not affected by overexpression of TRAF2 (Supplemental Fig. 4), which confirmed the specificity of these results. These results demonstrate that TRAF2 and TRAF5 negatively regulate the IL-6–IL-6R–gp130–STAT3 axis in CD4$^+$ T cells.

Finally, to identify more exactly the role of TRAF2 for Th17 development, naive CD4$^+$ T cells from wild-type or *Traf5*$^{-/-}$ OT-II mice (which have transgenic expression of an OVA-specific TCR) were stimulated with Ag OVA peptide (amino acids 323–339) and splenic APCs from wild-type B6 mice in the presence of IL-6, and transduced with a retrovirus vector encoding GFP and shTRAF2 to assess whether TRAF2 has an inhibitory function for Th17 development in wild-type or *Traf5*$^{-/-}$ CD4$^+$ T cells. shTRAF2 significantly enhanced the production of IL-17A relative to that elicited by control nontargeting shRNA both in wild-type and in *Traf5*$^{-/-}$ T cells (Fig. 5). Interestingly, shTRAF2 further promoted the production of IL-17A in *Traf5*$^{-/-}$ T cells (Fig. 5A). The increased IL-17A response was only detected in GFP$^+$ gated population but not in GFP$^-$ population (Fig. 5A), showing the specificity of TRAF2 knockdown.

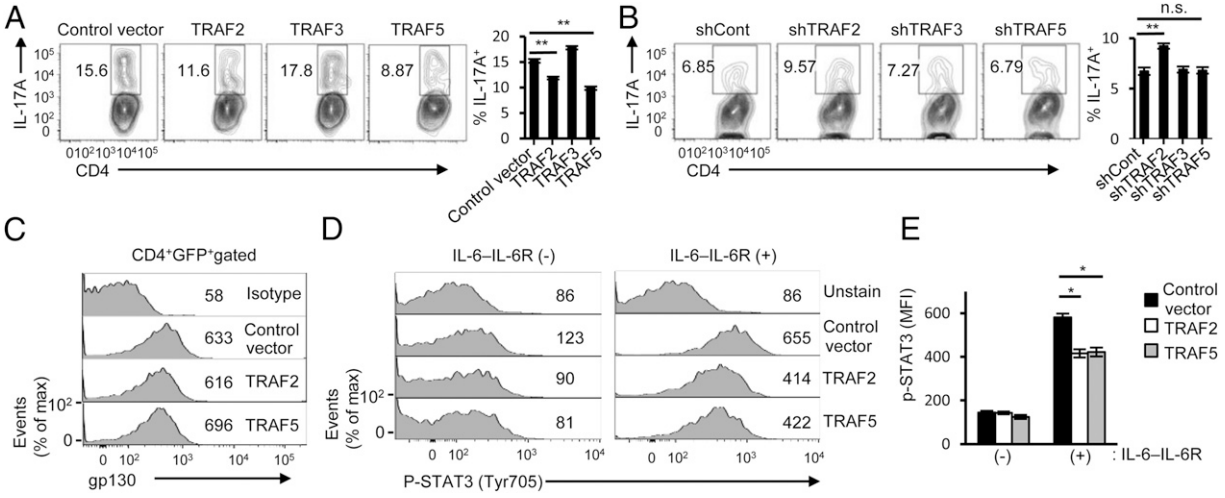

FIGURE 4. TRAF2 and TRAF5 negatively regulate Th17 development through antagonizing STAT3 activation. (**A** and **B**) Expression of IL-17A in activated CD4$^+$ T cells generated from wild-type naive CD4$^+$ T cells cultured for 12 h with anti-CD3 (1 μg/ml) and anti-CD28 (1 μg/ml) plus IL-2 (5 ng/ml) in the presence of blocking Abs to IFN-γ (1 μg/ml) and IL-4 (1 μg/ml), and then transduced with retroviral vector expressing GFP alone (control vector) or GFP and TRAF2, TRAF3, or TRAF5 (A) or retroviral vector expressing GFP and nontargeting shRNA (shCont) or GFP and shRNA that targets mRNA encoding TRAF2 (shTRAF2), TRAF3 (shTRAF3), or TRAF5 (shTRAF5) (B), followed 24 h later by the induction of Th17 differentiation for an additional 3 d through the addition of IL-6–IL-6R (30 ng/ml) and TGF-β1 (0.1 ng/ml), then restimulation for 5 h with PMA and ionomycin. Numbers adjacent to outlined areas indicate percentage IL-17A$^+$ cells among total GFP$^+$CD4$^+$ cells. (**C–E**) Flow cytometry of wild-type CD4$^+$ T cells activated for 12 h with anti-CD3 and anti-CD28 plus IL-2 in the presence of blocking Abs to IFN-γ and IL-4 as in (A) and (B), and then transduced with retroviral vector expressing GFP alone (control vector) or GFP and TRAF2 or TRAF5, followed 48 h later by resting activated T cells for an additional 48 h through addition of IL-2 (5 ng/ml), then serum starvation for 3 h and stimulated with IL-6–IL-6R (10 ng/ml) for 10 min. (C) Expression level of gp130 on rested GFP$^+$CD4$^+$ T cells described above. Isotype, isotype-matched control Ab. Numbers indicate mean fluorescence intensity of gp130. (D and E) Intracellular phosphorylated STAT3 (Tyr^{705}) levels in GFP$^+$ CD4$^+$ gated populations with (+) or without (−) IL-6–IL-6R stimulation. Numbers indicate mean fluorescence intensity (MFI) of p-STAT3. Data are mean values with SD of mean MFI of p-STAT3 from triplicate samples. Data are from one experiment representative of at least two independent experiments with similar results. *$p < 0.05$ (Student *t* test).

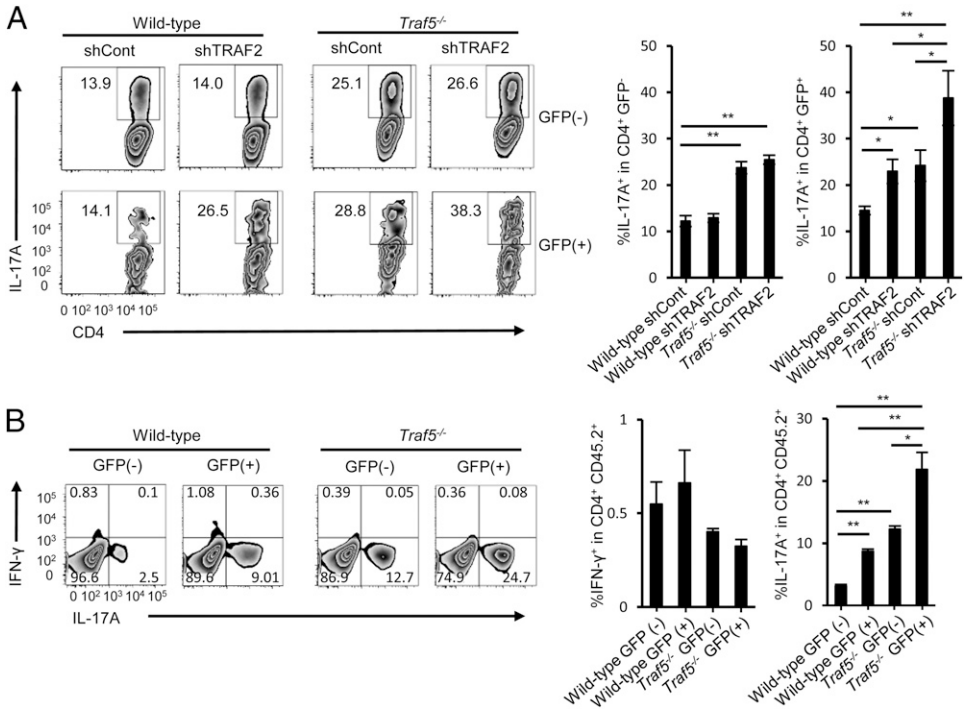

FIGURE 5. TRAF2 and TRAF5 synergistically limit Th17 differentiation both in vitro and in vivo. (**A**) Expression of IL-17A in activated CD4$^+$ T cells generated from wild-type or *Traf5*$^{-/-}$ naive OT-II CD4$^+$ T cells cultured for 12 h with 0.2 μM OVA peptide (amino acids 323–339) and irradiated wild-type B6 splenic APCs (after depletion of T cells) in the presence of IL-2 (5 ng/ml), IL-6–IL-6R (30 ng/ml), TGF-β1 (0.1 ng/ml) and blocking Abs to IFN-γ (2 μg/ml), IL-4 (2 μg/ml), and IL-12 (2 μg/ml), and then transduced with retroviral vector expressing GFP and nontargeting shRNA (shCont) or GFP and shRNA that targets mRNA encoding TRAF2 (shTRAF2), followed 4 d later by restimulation for 5 h with PMA and ionomycin. Numbers adjacent to outlined areas indicate percentage IL-17A$^+$ cells among GFP$^-$CD4$^+$ cells (*upper panels*) or GFP$^+$CD4$^+$ cells (*lower panels*). (**B**) Detection of IFN-γ– and IL-17A–producing OT-II (CD4$^+$CD45.2$^+$GFP$^-$ or CD4$^+$CD45.2$^+$GFP$^+$) cells obtained from the draining lymph nodes of CD45.1$^+$ host B6 mice given shTRAF2-transduced wild-type or *Traf5*$^{-/-}$ OT-II donor cells on day 0, followed by immediate immunization of the host with OVA protein in CFA and node harvest on day 7, then restimulation of the cells in vitro for 5 h with PMA and ionomycin. Numbers in quadrants indicate the percentage of total CD4$^+$ CD45.2$^+$GFP$^-$ (shTRAF2$^-$) or CD4$^+$CD45.2$^+$GFP$^+$ (shTRAF2$^+$) T cells represented by the subset of interest. Data are from one experiment representative of two independent experiments with similar results. *$p < 0.05$, **$p < 0.01$ (Student *t* test).

Next, to determine whether TRAF2 serves a similar function in vivo, we adoptively transferred wild-type or *Traf5*$^{-/-}$ OT-II T cells, which had been transduced with a vevtor containing GFP and shTRAF2, into congenic B6 mie, followed by s.c. immunization of the recipient mice with OVA protein in CFA. We then assessed recall IL-17 responses in donor GFP$^-$ or GFP$^+$ OT-II cells in the draining lymph nodes 7 d after immunization. As expected from in vitro results, shTRAF2 enhanced IL-17 but not IFN-γ production both in wild-type and in *Traf5*$^{-/-}$ OT-II cells (Fig. 5B). Therefore, TRAF2 has an antagonizing function in IL-6R signaling required for Th17 development in vivo. However, in contrast to TRAF5, TRAF2 showed a prolonged half-life in activated CD4$^+$ T cells (Fig. 1), and this resulted in the enhanced inhibitory activity for Th17 differentiation (Fig. 4B). Collectively, TRAF2 and TRAF5 have redundant and differential functions in the process of IL-6–mediated Th17 differentiation.

Binding of TRAF2 to gp130 antagonizes the recruitment of STAT3 to gp130

The antagonistic activity of TRAF5 for IL-6R signaling relies entirely on the TRAF-C domain (18), suggesting that TRAF2 employs the same mechanism as TRAF5 to inhibit the pathway. To explore the molecular mechanism by which TRAF2 inhibits IL-6R signaling, we transfected HEK293T cells to express both c-Myc–tagged gp130 and V5-tagged TRAF2 and immunoprecipitated proteins from cell lysates with anti-gp130. We found that TRAF2 was immunoprecipitated together with wild-type gp130 (1–917) (Fig. 6A).

TRAF5 recognizes TRAF-binding motifs, ^{776}SRSE^{779} and ^{788}EERPED^{793}, in gp130, implying that TRAF2 binds to the same region via its TRAF-C domain. Indeed, gp130 mutants with a deletion (Δ774–798) and alanine substitutions (^{776}AAAA^{779} or ^{788}AARPAA^{793}) showed considerably diminished binding to TRAF2 relative to that of wild-type gp130 (Fig. 6A). Additionally, TRAF-C domain was critical for the binding of TRAF2 to gp130 (Fig. 6B). These results demonstrate that TRAF-C domains of TRAF2 and TRAF5 recognize the same TRAF-binding motifs in gp130.

To examine the mechanism by which TRAF2 inhibits the IL-6R signaling, BAF-gp130 cells with control vector or shTRAF2 or shTRAF5 were stimulated with IL-6–IL-6R, and then the activated gp130 was pulled down to assess the binding between gp130 and STAT3. The STAT3 recruitment to gp130 was increased by the expression of shRNA that targets TRAF2 or TRAF5 (Fig. 6C), supporting the above results that both TRAF2 and TRAF5 are inhibitory for STAT3 activation mediated by IL-6 (Figs. 3C, 3D, 4D, 4E). In conclusion, we have found a novel regulatory function of TRAF2 in IL-6R signaling, and our data demonstrate a cooperative and differential regulatory mechanism that is driven by TRAF2 and TRAF5 expressed in developing Th17 cells.

Discussion

In this study, we show that not only TRAF5 but also TRAF2 has an inhibitory role for IL-6R signals needed for Th17 development. TRAF2 shares the TRAF-binding motifs in gp130 with TRAF5 and inhibits the recruitment of STAT3 to gp130, demonstrating a

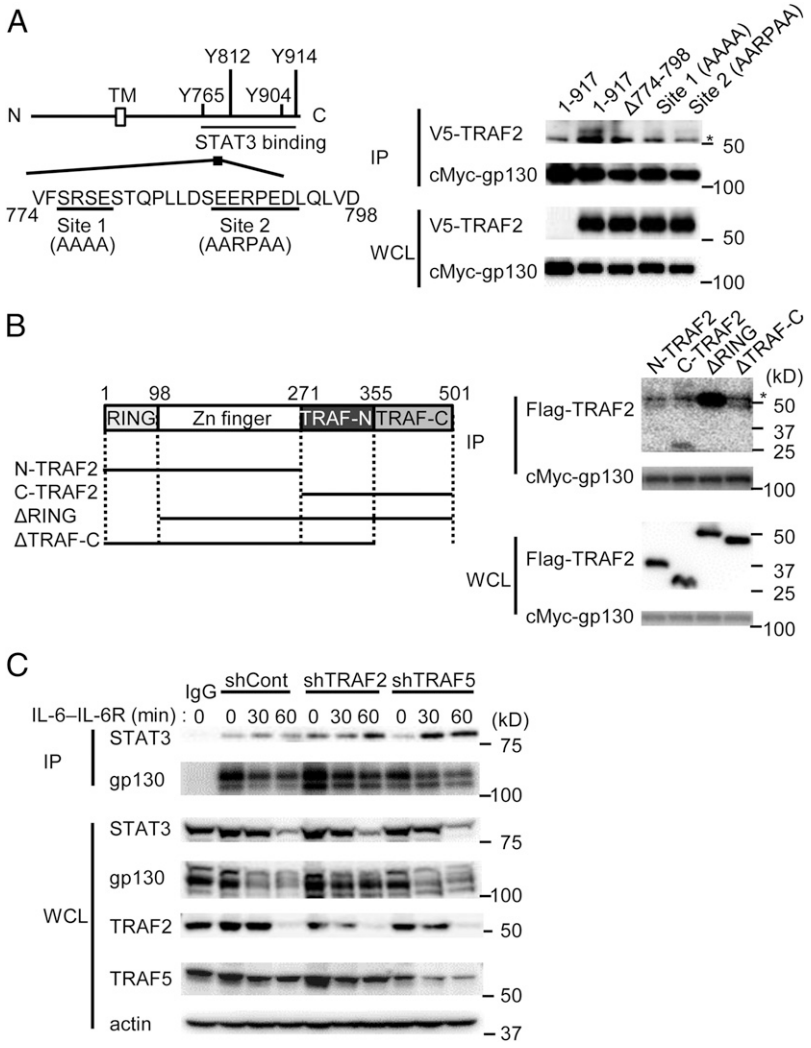

FIGURE 6. Binding of TRAF2 to gp130 via the C-terminal TRAF domain inhibits the IL-6–mediated recruitment of STAT3 to gp130. (**A**) Immunoassay of HEK293T cells transduced with plasmids encoding c-Myc–tagged gp130 mutants with a deletion in the cytoplasmic region in position 774–798 (Δ774–798, ■) or with substitution of alanine for other amino acids in TRAF-binding sites 1 and 2 (site 1 [AAAA] and site 2 [AARPAA]) and cotransfected to express V5-tagged TRAF2, followed by immunoprecipitation (IP) of proteins from lysates with anti-gp130 and immunoblot analysis with anti-V5 or anti-c-Myc. An asterisk indicates an H chain. (**B**) Immunoprecipitation of gp130 from lysates of HEK cells transiently transfected with plasmid vectors encoding c-Myc–tagged gp130 together with Flag-tagged TRAF2 mutants, N-TRAF2, C-TRAF2, ΔRING, or ΔTRAF-C, followed by immunoblot analysis with anti–c-Myc or anti-Flag. An asterisk indicates an H chain. (**C**) Immunoassay of BAF-gp130 cells stably transduced to express nontargeting shRNA (shCont) or shRNA that targets mRNA encoding TRAF2 (shTRAF2) or TRAF5 (shTRAF5) stimulated for indicated times with IL-6–IL-6R (500 ng/ml), followed by immunoprecipitation of proteins from lysates with anti-gp130 or control IgG and immunoblot analysis with indicated Abs. Data are from one experiment representative of two independent experiments with similar results. WCL, whole-cell lysate.

functional redundancy between TRAF2 and TRAF5. However, the inhibition kinetics of TRAF2 for IL-6 signaling is different from that of TRAF5. TRAF5 expressed in naive $CD4^+$ T cells is rapidly downregulated upon TCR triggering, and thus there is only a narrow window of time for the inhibition for IL-6 signaling. Alternatively, TRAF2 expression is stably detected at 0 and 3 d during the course of T cell activations, suggesting that TRAF2 can continuously suppress IL-6R signaling as long as both gp130 and IL-6R are expressed. Our results highlight a novel regulatory mechanism of TRAF2 and TRAF5 that is critical for the lineage commitment of Th17 cells.

Our data show that amounts of TRAF2 and TRAF5 are differentially controlled in developing $CD4^+$ T cells. Naive $CD4^+$ T cells had higher amounts of TRAF5 protein and mRNA, and most TRAF5 disappeared within 4 h after anti-CD3 stimulation, whereas TRAF2 protein and mRNA were stably expressed in $CD4^+$ T cells even after stimulation with anti-CD3 in the presence or absence of anti-CD28 and IL-6. Additionally, we examined changes in mRNAs of TRAF family molecules in wild-type naive $CD4^+$ T cells during the first 4 h after stimulation with anti-CD3/CD28 and realized that the expression profile could be categorized into three patterns: unchanged, TRAF2; upregulated, TRAF1, TRAF3, TRAF4, and TRAF6; and downregulated, TRAF5. This analysis implies that TRAFs are controlled by specific regulatory mechanisms in activated $CD4^+$ T cells. Especially, the expression profile of TRAF5 is totally different from other TRAFs, that is, TRAF5 expression is under the control of TCR signaling. It has been reported that the expression of TRAFs is negatively controlled by microRNAs (26–29) and the ubiquitin system (30–32). Additional study is required to understand how TCR signaling specifically controls downregulation of TRAF5 in $CD4^+$ T cells.

Betz and Müller (33) reported that anti-CD3/CD28 decreased the cell surface gp130 and IL-6R in splenic $CD4^+$ T cells and that this did not relate to IL-6–mediated receptor internalization, which indicates that TCR/CD28 limits the activity of the IL-6R complex. In this study, we could confirm this phenotype and further characterize the functional outcome of downmodulation of this receptor in relationship to TRAF expression and Th17 development: 1) naive $CD4^+$ T cells express gp130 and IL-6R on the surface, and TCR/CD28 signaling downregulates the expression of both receptor proteins, thereby decreasing the sensitivity to IL-6 that requires Th17 differentiation; 2) gp130-associated TRAF5 in naive $CD4^+$ T cells inhibits initial IL-6R signaling activity; 3) TRAF5 downregulation by TCR/CD28 is kinetically faster than IL-6R/gp130 downregulation by TCR/CD28, and thus the early TCR/CD28 signaling would promote IL-6–mediated Th17 differentiation; and 4) TRAF2 is not decreased by TCR/CD28, and thus the inhibitory effect of TRAF2 for IL-6–driven Th17 differentiation is relatively long-lasting compared with that of TRAF5. Taken together, our results clearly show that TCR/CD28-mediated T cell activation spatiotemporally modulates the expression balance of TRAF2, TRAF5, gp130, and IL-6R in $CD4^+$ T cells and that this balance critically influences reactivity of primed $CD4^+$ T cells to instructive IL-6R signals that are required for Th17 development.

NF-κB–inducing kinase (NIK) is an important signaling factor for Th17 differentiation (34), and TRAF2 and TRAF3 limit NIK activity via its ubiquitin-dependent degradation (32, 35, 36). These studies suggest that TRAF2 and TRAF3 negatively regulate Th17 differentiation through NIK degradation. In our study, however, although expression of TRAF2 was inhibitory for Th17 differentiation, expression of TRAF3 did not alter the sensitivity in IL-6R signaling and the IL-6–mediated Th17 development, which indicates that the TRAF3–NIK axis does not contribute to Th17 development. Thus, it is unlikely that TRAF2 and TRAF5 limit the Th17 differentiation through NIK degradation.

Collectively, we have uncovered a novel regulatory mechanism of Th17 differentiation that is critically controlled by gp130-associated TRAF2 and TRAF5. In the future, it will be important to explore the molecular mechanism by which TCR/CD28 signals regulate expression of TRAFs and to understand how the signaling crosstalk mediated by TRAFs regulates the lineage-commitment process of $CD4^+$ T cells that impacts protective immunity and pathogenesis of inflammatory and autoimmune diseases.

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Disclosures

The authors have no financial conflicts of interest.

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