TRAF5-mediated Lys-63-linked Polyubiquitination Plays an Essential Role in Positive Regulation of RORγt in Promoting IL-17A Expression*

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Background: RORγt is required for Th17 cell function and differentiation.

Results: TRAF5 stabilizes RORγt by ubiquitination and augments RORγt-mediated transcriptional expression of IL-17A.

Conclusion: TRAF5 is a positive regulator for RORγt.

Significance: TRAF5 could be a novel target for modulating RORγt-mediated inflammation and autoimmune diseases, including systemic lupus erythematosus.

Retinoid-related orphan nuclear receptor γt (RORγt) is a key transcription factor for the development and function of Th17 cells. In this study, we show that tumor necrosis factor receptor-associated factor 5 (TRAF5), known as an E3 ubiquitin protein ligase and signal transducer, interacts with and ubiquitinates RORγt via Lys-63-linked polyubiquitination. TRAF5 stabilizes the RORγt protein level depending on its RING finger domain. Depletion of TRAF5 in Th17 cells destabilizes RORγt protein and down-regulates Th17-related genes, including IL-17A, an inflammatory cytokine involved in pathogenic mechanisms of several autoimmune diseases such as systemic lupus erythematosus. Moreover, up-regulation of the TRAF5 mRNA level was found in systemic lupus erythematosus patient CD4+ T cells. Our findings reveal a direct link between TRAF5-mediated ubiquitination and RORγt protein regulation, which may aggravate inflammatory progress and provide new therapeutic drug targets for autoimmune diseases.

Th17 cells, which have attracted widespread attention as a subset of CD4+ T cells, contribute to the pathogenesis of multiple autoimmune and inflammatory diseases, such as systemic lupus erythematosus (SLE),4 inflammatory bowel disease, rheumatoid arthritis, and ankylosing spondylitis (1). Similar to Th1 and Th2 cells, Th17 cells express retinoic acid-related orphan nuclear receptor γt (RORγt) as the master regulator, orchestrating the differentiation, maintenance, and function of the Th17 lineage (2). RORγt is an isoform of RORγ with a distinctive N terminus and directs the transcription of the Th17-related cytokines IL-17A and IL-17F, which work as effector molecules of Th17 by inducing inflammatory cytokines and chemokines (2). Despite its significance for Th17 cells, the regulation of RORγt stability is still unclear.

Several E3 ubiquitin protein ligases and deubiquitinating enzymes have been confirmed to be important in the regulation of Th17 cell differentiation and function. Tumor necrosis factor receptor-associated factor 3 (TRAF3) is a negative regulator of IL-17R signaling (3), whereas TRAF5 and TRAF6 are essential for IL-17R signaling (4). The ubiquitin-specific protease USP18 plays a key role for T cells in the progres of differentiating into Th17 cells (5). USP25 could remove the Lys-63-linked ubiquitination in TRAF5 and TRAF6 mediated by Act1 and inhibit IL-17R signaling and inflammation (6).

However, these aforementioned studies did not focus on the ubiquitination and deubiquitination of RORγt, which is considered to be a meaningful posttranscriptional modification. Our previous research has revealed that both USP17 and USP4 could prevent RORγt degradation and modulate Th17 cell function through reducing Lys-48-linked polyubiquitination (7, 8).

Originally, TRAF5 was identified as a signaling adaptor involved in the CD40, nucleotide binding oligomerization domain-like receptor, retinoic-acid inducible gene I like receptor, and also IL-17 receptor (IL-17R) signaling pathways. In addition to its role as an adaptor protein, TRAF5 could also act

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4 The abbreviations used are: SLE, systemic lupus erythematosus; rh, recombinant human; TRAF, tumor necrosis factor receptor-associated factor; SLEDAI, systemic lupus erythematosus disease activity index.

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as E3 ubiquitin ligase containing an N-terminal RING finger domain to exert catalytic activity (9).

In this paper, we investigate the role of TRAF5 in RORγt regulation. TRAF5 was identified as an E3 ubiquitin-protein ligase of RORγt by stabilizing and facilitating Lys-63-linked ubiquitination of RORγt via its RING finger domain. Furthermore, TRAF5 could interact with RORγt and promote RORγt-mediated IL-17A transcription.

**Experimental Procedures**

**Plasmids, Antibodies, and Reagents—** RORγt and TRAF5 and their corresponding truncations were amplified from HEK293T cells or human peripheral blood mononuclear cell cDNA and then cloned into the pIPIFLAG2 or pIPMyc2 vectors. The shRNA expression vector pLKO.1 was purchased from Addgene. The plasmids Δ8.9 and vesicular stomatitis virus glycoprotein were gifts from Ke Lan (Institut Pasteur of Shanghai, Chinese Academy of Sciences). The antibodies used in this study included the following: anti-RORγt (catalog no. H-190, Santa Cruz Biotechnology), anti-TRAF5 (catalog no. H-257, Santa Cruz Biotechnology), anti- Ubiquitin (catalog no. P4D1, Santa Cruz Biotechnology), anti-HA (catalog no. F-7, Santa Cruz Biotechnology), anti-Myc (catalog no. 9E10, Santa Cruz Biotechnology), anti-FLAG (catalog no. M2, Sigma-Aldrich), and anti-β-actin (catalog no. C1213, Tianjin Sungen Biotech). Mouse IgG was from Millipore. Protein A/G Plus-agarose (catalog no. sc-2003) was purchased from Santa Cruz Biotechnology. MG132 3 h before harvesting. Cells were washed with ice-cold PBS and then lysed in urea buffer (pH 8.0) (10 mM Tris (pH 8.0), 8 M urea, 100 mM NaHPO₄, 0.2% Triton X-100, and 10 mM imidazole) for 30 min. The lysates were incubated with nickel-nitritotriacetic acid beads (catalog no. 30210, Qiagen) for 3 h at room temperature. The beads were washed three times in urea buffer (pH 8.0) before incubation. After 3 h of incubation, the beads were washed twice in urea buffer (pH 8.0), twice in urea buffer (pH 6.3), 3 M urea, 100 mM NaHPO₄, 0.2% Triton-100, and 10 mM imidazole), and once in wash buffer (20 mM Tris (pH 8.0), 100 mM NaCl, 20% glycerol, 1 mM dithiothreitol, and 10 mM imidazole). Beads were boiled in 2× sample loading buffer and analyzed by SDS-PAGE.

**Luciferase Reporter Assay—** The -600 to 0 region of the human IL-17A promoter with one RORγt binding site was cloned into the pGL3-Basic vector to generate the pGL3-IL-17a-Luc reporter construct. To analyze the effects of TRAF5 on RORγt in terms of IL-17A promoter activity, the IL-17a luciferase reporter plasmid was co-transfected with a β-gal-luciferase reporter into HEK293T cells. After 48 h, the cells were lysed, and luciferase reporter assays were performed using the Dual-Luciferase reporter kit (Promega).

**Quantitative Real-time PCR—** Total RNA was extracted from whole cells as well as CD4⁺ T cells from SLE patients and healthy controls using TRIzol reagent (Invitrogen) following the instructions of the manufacturer. cDNA was synthesized using a reverse transcriptase kit (TaKaRa), followed by quantitative RT-PCR analysis (SYBR Green, TaKaRa). The sequences of the used primers were as follows: IL-17A, 5'-accaatccccaaaa-ggtcctc-3' (forward) and 5'-ggagagacagagttctggtg-3' (reverse); IL-17F, 5'-ctctccctctgtaaatcact-3' (forward) and 5'-accagaccct-tttcaacttg-3' (reverse); IL-21, 5'-aggctaacacggccatca-3' (forward) and 5'-ttgccctgcaacacgccga-3' (reverse); IL-23, 5'-tttccacaaacacacatc-3' (forward) and 5'-atgctgatgatgtttccc-3' (reverse); RORC, 5'-ctgtgacgaggaggagagag-3' (forward) and 5'-aggcttgctgagggggtgttg-3' (reverse); TRAF5, 5'-aactgccca- caatcagc-3' (forward) and 5'-taggttagttagctgctg-3' (reverse); and β-actin, 5'-cttcctgctccctcctc-3' (forward) and 5'-tgagggctgtgatctc-3' (reverse).

**Lentiviral Constructs and Infection—** The shRNA oligos were cloned into the lentiviral vector pLKO.1 with a resistant of puromycin. Then shTRAF5, Δ8.9 and vesicular stomatitis virus glycoprotein were transfected into HEK293T cells. Viral super-
natants were harvested after 48 h. Th17 cells were incubated with viral supernatants containing 8 μg/ml of Polybrene overnight. The viral supernatants were replaced with fresh medium on day 2. Puromycin was added to select the cells 2 days post-infection. The following shRNA sequences were used: shCK, 5′-caacaagatgaagaccaaa-3′; shTRAF5-1, 5′-gatgtaatgccaagttattc-3′; shTRAF5-2, 5′-ggctgtgctgtaacggataaa-3′; and shTRAF5-3, 5′-cagtgtctcgggcactaaa-3′.

CD4+ T Cell Isolation—Human peripheral blood was collected from SLE patients who met the American College of Rheumatology criteria for SLE as well as from healthy controls. Patients were between 20 and 55 years old and were recruited from the Rheumatology Department of Huashan Hospital (Shanghai, China). The patients were divided into two groups according to the disease activity index (SLEDAI): an inactive group (SLEDAI < 8) and an active group (SLEDAI ≥ 8). CD4+ T cells were isolated from whole blood using a human CD4+ T cell enrichment mixture (StemCell Technologies).

Statistical Analyses—Data are presented as mean ± S.D. Student’s t test was used for comparisons on GraphPad Prism 5.0, with p < 0.05 considered statistically significant.

Results

TRAF5 Up-regulates RORγt-mediated IL-17a Transcription—To explore whether any member of the TRAF family could regulate RORγt-mediated IL-17a transcriptional activity, the effects of five TRAF family members were tested by co-transfection of each individual FLAG-TRAF together with Myc-RORγt and the luciferase reporter construct containing the IL-17a promoter into HEK293T cells. TRAF5 significantly
up-regulates RORγt-mediated IL-17a transcription compared with the control (Fig. 1A). The positive regulation of IL-17a transcription by TRAF5 was dose-dependent (Fig. 1B), whereas that of TRAF5ΔN90 (TRAF5ΔN) was not (Fig. 1C). TRAF5ΔN is a truncated form of TRAF5 lacking the N-terminal RING finger domain.

**TRAF5 Interacts with and Stabilizes RORγt**—We next verified our hypothesis that the up-regulation of IL-17a transcription activity via TRAF5 may be on the basis of the interaction between TRAF5 and RORγt. First, HEK293T cells were introduced with FLAG–TRAF5s and Myc–RORγt, followed by co-immunoprecipitation assay. The result revealed that TRAF5 could interact with RORγt, especially in the TRAF family (Fig. 2A). Second, expression vectors containing Myc–RORγt and FLAG–TRAF5 were co-transfected and ectopically expressed in HEK293T cells for co-immunoprecipitation. A positive interaction was observed between TRAF5 and RORγt, and the RING finger domain of TRAF5 was dispensable for the interaction (Fig. 2B). In addition, the physical interaction was demonstrated by endogenous immunoprecipitation in human primary Th17 cells. We also tested different time points to check the interaction between TRAF5 and RORγt and found that a T cell receptor signal was necessary for the interaction (Fig. 2C).

Considering the aforementioned results, we then assessed the effects of TRAF5 on RORγt stability by TRAF5. Myc–RORγt and FLAG–TRAF5 or a controlled FLAG vector were transfected into HEK293T cells. It was observed that the protein level of RORγt was up-regulated by TRAF5 in a dose-dependent manner whereas TRAF5ΔN was not (Fig. 2D). To confirm these results, we treated HEK293T cells introduced with or without TRAF5 or TRAF5ΔN with the protein synthesis inhibitor cycloheximide for the indicated times. The overexpression of TRAF5 effectively prolonged the half-life of RORγt protein but its TRAF5ΔN truncation did not (Fig. 2E). Taken together, these results showed that TRAF5 interacted with and stabilized RORγt.

**TRAF5 Promotes the Ubiquitination of RORγt**—Because RORγt could be polyubiquitinated (6) and TRAF5 is an E3 ubiquitin protein ligase containing a RING finger domain, we reasoned that the function of TRAF5 toward RORγt might depend on its ubiquitin ligase activity. To further identify the role of TRAF5 in the regulation of RORγt, we co-transfected Myc–RORγt and His-ubiquitin into HEK293T cells with or without FLAG–TRAF5 or TRAF5ΔN. These cells were treated with the proteasome inhibitor MG132 for 3 h after 2 days to stabilize the ubiquitinated proteins before they were lysed under denaturing conditions. Our analysis revealed that the RORγt polyubiquitination was facilitated significantly by wild-type TRAF5 but not by the catalytically inactive truncated TRAF5ΔN (Fig. 3A).

Different types of polyubiquitin chains with definitive function could be made up by ubiquitin monomers because there were seven lysine residues in a ubiquitin molecule (Lys-6, Lys-11, Lys-27, Lys-29, Lys-33, Lys-48, and Lys-63). So far, the ubiquitination via Lys-48 or Lys-63 linkage has been well characterized. For example, Lys-63-linked polyubiquitination could signal in various proteasome–independent pathways, including ribosomal protein synthesis and DNA repair. On the contrary, Lys-48-linked polyubiquitination usually functions in the canonical signal for proteasomal degradation (10). In our study, RORγt was co-expressed with the Lys-63 only or Lys-48 only ubiquitin mutant, followed by nickel-nitritoltriacetic acid pull-down assay under denaturing conditions, and more TRAF5-mediated ubiquitination of RORγt was observed under the former condition (Fig. 3B). The result was consistent with the previous one, where the ubiquitin mutant was changed to K63R or K48R (Fig. 3C). Therefore, our data indicate that TRAF5 ubiquitinates RORγt mainly through Lys-63 linkage, which depends on its activity. To find out under what physiological condition RORγt is ubiquitinated, we carried out different stimulations of primary human Th17 cells after anti-CD3 and anti-CD28 Abs treatment overnight. Under the condition of IL-6 + TGF-β (5 ng), the ubiquitination of RORγt was the most, which suggested an important role of IL-6 and TGF-β in promoting ubiquitination of RORγt (Fig. 3D).

**Knockdown of TRAF5 Decreases the Ubiquitination and Protein Level of RORγt**—As mentioned above, TRAF5 could mediate the protein stabilization and function of RORγt, suggesting that TRAF5 contributes to the development of Th17 cells. To determine whether TRAF5 is involved in the regulation of Th17 function, three shTRAF5-expressing plasmids were constructed and tested by overexpression in HEK293T cells, and they could knock down TRAF5 effectively, except shTRAF5-3 (Fig. 4A). We found that the protein level of RORγt and Th17-related cytokine gene transcription levels were reduced in TRAF5-deficient human primary Th17 cells, whereas the RORγt mRNA levels did not (Fig. 4B and C). In addition, the ubiquitination level of RORγt was decreased in the presence of shTRAF5 (Fig. 4B). These results indicate that TRAF5 is vital to the function of Th17 cells by mediating the stabilization and ubiquitination of RORγt.

**TRAF5 Expression Is Associated with Inflammation in Autoimmune Systemic Lupus Erythematosus**—Considering the stimulative role played by Th17 cells in the pathogenesis of SLE, we detected TRAF5γt, RORγt, and Th17-related cytokine gene expression levels in CD4+ T cells from SLE patients and healthy donors. The mRNA levels of the Th17 cell transcription factor RORγt and cytokines such as IL-17A, IL-17F, IL-21, and the surface receptor IL-23R all increased in the patients. However, only the differences in IL-17A and IL-17F levels were statistical significant. The increased TRAF5 levels also showed a significant difference between SLE patients and healthy donors (Fig. 5A). Moreover, the elevated expression of TRAF5 was positively correlated with that of IL-17A (Fig. 5B), and TRAF5 was highly expressed in active patients compared with inactive patients and healthy donors. Our data show that TRAF5 might be related to the function of the Th17 lineage in SLE.

**Discussion**

Although both RORγt and TRAF5 have been implicated in the pathogenesis of inflammation and autoimmune diseases, it has not been established whether there is a physical or functional link between the two. Our study shows that TRAF5, as a positive regulator of RORγt in the Th17 lineage, is an E3 ubiquitin protein ligase of RORγt.

RORγt, a major transcription factor in Th17 cells, promotes Th17 lineage development and exerts multifunctionality in the
immune response. However, little attention has been paid to the
posttranslational modification of RORγt. Our previous studies
have identified USP17 and USP4 as the deubiquitinating enzymes
of RORγt (7, 8). Furthermore, UBR5 and deubiquitinating enzyme
have been reported as a pair of antagonistic E3 ligase and deu-
biquitinating enzyme regulating the expression of RORγt (11).

**FIGURE 2. TRAF5 interacts with and stabilizes RORγt.** A. TRAF family members and Myc-RORγt were co-transfected into HEK293T cells. Cell lysates were
immunoprecipitated (IP) with anti-Myc antibody. IB, immunoblot. B, Myc-RORγt and FLAG-TRAF5 or FLAG-TRAF5ΔN were co-transfected into HEK293T cells.
Cell lysates were immunoprecipitated with either anti-Myc or anti-FLAG antibody. C, naïve CD4+ T cells were polarized under Th17 conditions, and then
Th17 cells were harvested and lysed with radioimmune precipitation assay buffer. The cells lysate was immunoprecipitated with anti-TRAF5 antibody or a
rabbit IgG control. Th17 cells were stimulated by anti-CD3 and anti-CD28 antibodies after resting for 1 day, and cells were collected at 0, 1, 4, and 8. D, HEK293T
cells were co-transfected with Myc-RORγt and the indicated doses of FLAG-TRAF5 or its mutant (0, 0.4, 0.8, and 1.2). The statistical curve was determined as
shown. E, Myc-RORγt with or without FLAG-TRAF5 or TRAF5ΔN was transfected into HEK293T cells. The half-life of RORγt in cycloheximide (CHX)-treated cells
was analyzed as shown. Ctrl, control. Data represent at least three independent experiments.
Here we revealed that TRAF5 could interact with and stabilize RORγt. TRAF5 may increase RORγt-dependent IL-17α promoter transcriptional activity. Then, as an E3 ubiquitin protein ligase, TRAF5 could facilitate Lys-63-linked polyubiquitination of RORγt. Our results suggest a new potential mechanism by which TRAF5 positively regulates RORγt in Th17 cells.

Since its discovery, the function of TRAF5 has remained elusive despite intensive studies of the subject. More and more studies have shown that TRAF5 has both inflammatory and proinflammatory effects. In the infected pathological state, the fact that CD8+ T cells from Traf−/− mice were unresponsive points out the important function of TRAF5 in enhancing T cell expansion (12). TRAF5 also has vital role in the glucocorticoid-induced TNFR signaling pathway, which would activate T cells (13). On the other hand, some studies contribute to the conclusion that TRAF5 can act as an anti-inflammatory factor. Researchers found that naïve CD4+ T cells from Traf−/− mice tend to differentiate toward Th17 cells (14), whereas B lymphocytes from Traf−/− mice could produce more cytokines.
including IL-6, IL-10, and TNF-α, after Toll-like receptor stimulation (15).

It has been well established that TRAF5 is involved in the pathogenesis of several inflammatory and autoimmune diseases. The expression levels of TRAF5 increase in patients suffering from inflammatory bowel disease, and there is a weak correlation between TRAF5 and endoscopic disease activity index in patients with ulcerative colitis (16). Also, TRAF5 has been identified as a strong candidate for the rheumatoid arthritis susceptibility gene in the British population (17), and it is involved in the development of acute anterior uveitis (18), according to genetic polymorphism analysis in Han Chinese. In addition, cardiac hypertrophy and fibrosis are aggravated in Traf−/− mice compared with wild-type mice, suggesting that TRAF5 is a protective factor for the disease (19). We also confirmed a significant increase of TRAF5 in CD4+ T cells from patients with SLE, leading to the hypothesis that TRAF5 might drive the disease process by ubiquitinating RORγt under inflammatory conditions.

However, TRAF5 is involved in the Ox40/Ox40L pathway. Ox40L, also called tumor necrosis factor superfamily member 4 (TNFSF4), is highly expressed in peripheral blood mononuclear cells from patients with systemic lupus erythematosus compared with healthy controls (20). TNFSF4 has been identified as a risk factor of SLE because upstream of it was a susceptibility locus in SLE (21). Furthermore, TNFSF4 is associated with renal disorder in Chinese SLE patients (22). Considering the interaction between TRAF5 and Ox40 (23), we could not ignore the possibility that the overexpression of TRAF5 is partly attributable to the increase of Ox40/Ox40L in SLE.

Traditionally, it is well known that E3 ubiquitin protein ligases promote the degradation of target proteins. For example, Stub1 negatively modulates Foxp3 via promoting its Lys-48-linked polyubiquitination (24). Parkin mediates Lys-63-linked polyubiquitination of UCH-L1 and promotes UCH-L1 degradation by the autophagy-lysosome pathway (25). Unlike the conventional mechanism, our findings identify TRAF5 as a positive regulator of RORγt. The reason for stabilization is that TRAF5 might mediate more Lys-63-linked polyubiquitination than Lys-48-linked polyubiquitination of RORγt or that TRAF5 might simultaneously promote another lysine residue-linked ubiquitination of RORγt that we did not test.

In summary, our results suggest that TRAF5-mediated RORγt ubiquitination and stabilization could regulate immune disorder, especially in Th17-specific inflammation. Therefore, appropriately low TRAF5 levels may contribute to RORγt deg-
radiation and attenuated inflammation. Moreover, our study not only sheds light on the regulation of the Th17 lineage by ubiquitination but also provides a new explanation of the role of Th17 cells and potential therapeutic targets for autoimmune diseases.

Author Contributions—X. W. and J. Y. designed, performed, and analyzed the experiments shown in the figures. L. H. constructed the RORγt and IL-17a luciferase reporter plasmids. L. H. and K. Z. collected samples from SLE patients and healthy donors. Q. W., L. B., and Z. L. provided technical assistance and helped with the preparation of the manuscript. L. L. and B. L. conceived and coordinated the study, analyzed and interpreted data, and wrote the paper. All authors reviewed the results and approved the final version of the manuscript.

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