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Cutting Edge: Ubiquitin-Specific Protease 4 Promotes Th17 Cell Function under Inflammation by Deubiquitinating and Stabilizing RORγt

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RORγt is a key transcription factor that controls the development and function of inflammatory Th17. The mechanisms that regulate RORγt stability remain unclear. We report that Th17 cells highly express the deubiquitinase ubiquitin-specific protease (USP)4, which is essential for maintaining RORγt and Th17 cell function. Inhibition of the catalytic activity of USP4 with vialinin A, a compound derived from Chinese traditional medicine, dampened Th17 differentiation. USP4 interacted and deubiquitinated K48-linked polyubiquitination of RORγt, thereby promoting RORγt function and IL-17A transcription. Interestingly, TGF-β plus IL-6 enhanced USP4-mediated deubiquitination of RORγt. Moreover, USP4 and IL-17 mRNA, but not RORγt mRNA, were significantly elevated in CD4+ T cells from patients with rheumatic heart disease. Thus, USP4 could be a novel therapeutic target for the treatment of Th17-modulated autoimmune diseases. *The Journal of Immunology, 2015, 194: 4094–4097.

The differentiation of Th17 cells plays a critical role in the development of autoimmune diseases, such as rheumatic heart disease (RHD) (10). TGF-β regulates Th17 cells at a low concentration, and IL-6 promotes Th17 skewing (11, 12). Ubiquitin-specific protease (USP)4 was identified as a proto-oncogene, and TGF-β could mediate USP4 nuclear-to-cyttoplasmic transport (13). Recently, studies showed that IFNs induce E3 ligase Ro52−/− mice to promote tissue inflammation by deregulating the IL-23–Th17 pathway (14); Ro52 is the E3 ligase of USP4 (15). Vialinin A, a terphenyl compound originally isolated from the Chinese mushroom, exhibits effective anti-inflammatory activity, is used to treat rheumatoid arthritis, and inhibits USP4 DUB activity at low concentrations (15), suggesting that USP4 may play an important role in autoimmune diseases, which provided the impetus for this study.

In this study, we identified USP4 as a DUB of RORγt during inflammation and found that the inhibition of the catalytic activity of USP4 with vialinin A affects Th17 cell differentiation, which reveals an alternative to that previously reported small molecule halofuginone in modulating Th17 cell function (16).

Materials and Methods

Abs and reagents

Vialinin A and all of the cytokines used in this study were purchased from R&D Systems. The following Abs were used for immunoprecipitation (IP) and immunoblotting (IB): anti–T-bet (eBioscience), anti-RORγt, and anti-ubiquitin (Santa Cruz). Abs for USP4, GATA3, FOXP3, GAPDH, and β-actin were previously described (13, 17–19). Full-length USP4 was cloned from human PBMCs.

Real-time PCR and knockdown assay

Primers for real-time PCR were used as follows: IL-17A forward: 5'-ACCAATCCCAAAAGGTGCCTC-3'; IL-17A reverse: 5'-GGGGACAGA-GTTCATGTGGT-3'; IL-17F reverse: 5'-CCCCTGCCGAGAT-TCACCT-3'; IL-17F reverse: 5'-ACCAAGCACCTTCTCCAACTG-3'; USP4, GATA3, T-bet, RORγt, and GAPDH primers, as well as the short hairpin RNA (shRNA) target sequences, were used as previously described (13, 19, 20).

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Luciferase reporter assay

The −600 to 0 region of the IL-17A promoter containing an RORγt binding site was cloned into the pGL3-basic vector, and a luciferase assay was performed, as previously described (19).

His pull-down assay

His pull-down assays were performed as previously described (20).

Th cell polarization

Th17 cell polarization was conducted as follows. In brief, human naive T cells were isolated from healthy donor PBMCs and activated with anti-CD3/CD28 beads (1:1), TGF-β (1 ng/ml), IL-6 (1 ng/ml), IL-23 (100 ng/ml), and IL-1β (10 ng/ml) for 7 d. Th1 and Th2 induction were performed as previously described (19). For Western blotting, 1 × 10^6 cells were loaded onto SDS-PAGE gels, and 0.5 × 10^6 cells were used for flow cytometry.

Results and Discussion

USP4 promotes Th17 differentiation

Western blotting and real-time PCR analysis of different Th subsets (polarized from naive T cells) showed that the protein and mRNA levels of USP4 are highest in Th17 cells (Fig. 1A, Supplemental Fig. 2A). Given that RORγt is important to Th17 cell development, we aimed to determine the function of USP4 in Th17 cell differentiation using the USP4-specific inhibitor vialinin A (USP4i) (15). Upon treatment of human

FIGURE 1. USP4 promotes Th17 differentiation. (A) Human naive CD4+ T cells were differentiated into Th0, Th1, Th2, and Th17 cells. RORγt and USP4 levels were visualized by IB. (B) Human naive CD4+ T cells were treated under Th17 cell-polarization conditions, with or without a USP4 inhibitor (vialinin A; 1–2 μM). (C) RORγt and IL-17A levels were detected by flow cytometry and IB. (D) USP4 was knocked down in human Th17 cells using shRNA-bearing lentivirus and then selected for 7 d. (E) Endogenous RORγt and USP4 levels were visualized by IB and flow cytometry. All data are representative of at least three independent experiments.

FIGURE 2. USP4 interacts with and stabilizes RORγt. (A) Human naive T cells were isolated and polarized to Th17 in vitro then IP was carried out and analyzed by IB. (B) FLAG-RORγt, with or without HA-USP4 or C311A, were transfected into HEK293 T cells and treated with cycloheximide (CHX) (20 μg/ml). The RORγt level was detected with anti-FLAG Ab. (C) HEK293 T cells were transfected with FLAG-RORγt, HA-USP4, or His-ubiquitin (WT, 48K, and 63K) and treated with 20 μM MG132 for 3 h before harvest. Pull-down using Ni-NTA beads; ubiquitinated RORγt was visualized by IB using anti-FLAG Ab. (D) USP4 was knocked down in human Th17 cells using shRNA-bearing lentivirus. IP with RORγt Ab and endogenous RORγt ubiquitination level was visualized by IB. (E) Th17 cells were stimulated with anti-CD3 and anti-CD28 Abs overnight in fresh medium and treated with MG132 (5 μM) and USP4i (2 μM) for 8 h. IP was performed, and the results were visualized as in (D). Data are representative of at least three independent experiments.
 naïve T cells, with or without USP4i, during polarization, we found that both RORγt and IL-17A levels decreased significantly with 1–2 μM treatment compared with the mock control (Fig. 1B, 1C), suggesting that USP4 plays an important role in Th17 cell development. To further examine the activity of USP4 in Th17 function, we knocked down USP4 with an shRNA-bearing lentivirus and then tested at the protein level for RORγt expression. RORγt protein level decreased significantly, and IL-17 transcription was substantially downregulated (Fig. 1D, 1E), suggesting the likely possibility that USP4 affects Th17 differentiation through stabilization of RORγt at the protein level.

**USP4 interacts with and stabilizes RORγt**

To investigate further how USP4 affects Th17 cell differentiation, we first performed a reciprocal IP experiment; the results showed a clear interaction between RORγt and USP4 (wild-type [WT] or catalytically inactive mutant C311A) (Supplemental Fig. 1A, 1C). Then we established a Jurkat T cell line stably expressing FLAG-tagged RORγt (FLAG-RORγt) and visualized endogenous USP4 and FLAG-RORγt by immunostaining. The colocalization of both proteins was observed in the nucleus (Supplemental Fig. 1B). We further found that the DNA-binding domain of RORγt is essential for the interaction with USP4 at its USP domain (Supplemental Fig. 1D, 1E). We then confirmed the endogenous interaction between USP4 and RORγt in primary human Th17 cells (Fig. 2A).

To determine whether USP4 is required for the stability of RORγt, we overexpressed FLAG-RORγt and various doses of hemagglutinin-tagged USP4 (HA-USP4) (WT or mutant C311A); a dose-dependent effect of USP4 on RORγt expression was observed (Supplemental Fig. 2B). Treatment of the cotransfected cells with cycloheximide showed a clear effect of USP4 on RORγt protein half-life, but its C311A mutant had no effect (Fig. 2B), supporting a role for USP4 in stabilizing RORγt at the posttranslational level. To investigate how USP4 affects RORγt stability, and given that USP4 is a DUB, we performed a pull-down assay in which the RORγt polyubiquitination level was reduced after cotransfection of WT USP4 but not the C311A mutant. This suggests that USP4 may stabilize RORγt by deubiquitination (Supplemental Fig. 2C). Moreover, because previous studies showed that Lys-48–linked ubiquitination is an important mechanism for protein degradation, we mutated all of the lysines of ubiquitin to arginines with the exception of Lys-48

**FIGURE 3.** TGF-β and IL-6 promote USP4-mediated enhancement of RORγt-mediated IL-17A transcription. (A) Human Th17 cells were stimulated with anti-CD3 and anti-CD28 Abs overnight in fresh medium and then stimulated with different cytokines for 8 h. The protein levels of RORγt and USP4 were visualized by IB. (B) USP4 was knocked down in FLAG-Jurkat and FLAG-RORγt–Jurkat T cells with lentivirus and then transfected with the IL-17A reporter. Cells were stimulated with HEK and ionomycin (P+I) overnight with fresh medium, with or without TGF-β (1 ng/ml) or TGF-β (1 ng/ml) and IL-6 (20 ng/ml) for 8 h, followed by analysis of IL-17A transcription by a luciferase reporter assay. (C) FLAG-Jurkat and FLAG-RORγt–Jurkat T cells were stimulated with PMA and ionomycin (P+I) overnight and then treated with TGF-β (1 ng/ml), IL-6 (20 ng/ml), and MG132 (5 μM), with or without USP4i (1 μM), for 8 h and analyzed as described in Fig. 2D. (D) CD4+ T cells were isolated from the peripheral blood of RHD patients and healthy controls (n = 18). Data are representative of at least three independent experiments. *p < 0.05, **p < 0.01.
Because USP4 had a clear effect on Th17 differentiation and function by stabilizing RORγt, we further determined whether USP4 affects Th17 function by promoting RORγt-mediated induction of IL-17 expression. We constructed an IL-17A promoter (−600 to 0) reporter vector and transfected this vector into FLAG-Jurkat or FLAG-RORγt–Jurkat T cells; under USP4i treatment, there was a decreased level of promoter activity after PMA and ionomycin activation mediated induction of IL-17 expression. We constructed different stimulations of Flag-RORγt and function by stabilizing RORγt because USP4 had a clear effect on Th17 differentiation and deubiquitination of K48-linked polyubiquitination.

These data indicate that USP4 stabilizes RORγt specific for USP4, we then treated the cells with USP4i; the polyubiquitination level of RORγt was affected (Fig. 3C). All of these data suggest that IL-6 upon USP4 inhibitor treatment, suggesting that USP4 function was affected (Fig. 3C). All of these data suggest that IL-6 plus TGF-β enhances USP4 activity to further support a role for USP4 deubiquitination of RORγt in Th17 cell differentiation. To further verify whether USP4 modulates Th17 function in vivo under inflammatory stimuli, we tested USP4 expression in CD4+ T cells from RHD patients. There was a significant increase in USP4, IL-17A, and IL-17F mRNA in these patients compared with healthy controls, indicating a positive correlation between USP4 mRNA and Th17 cell function in inflammatory disease (Fig. 3D). Interestingly, our data also indicate that the level of USP4 mRNA correlates significantly with IL-17F, but not IL-17A, mRNA within the patient population (Supplemental Fig. 2G).

Our study proposes a previously uncharacterized regulation of RORγt through deubiquitination by USP4. Interestingly, we reveal a new mechanism for TGF-β– and IL-6–mediated regulation of Th17 differentiation by promoting USP4 function. We also reveal how inhibition of USP4 catalytic activity by viliain A impairs Th17 cell differentiation, as well as a positive correlation between USP4 mRNA and Th17 cell function in RHD. Our data suggest that USP4 could be considered a novel drug target to inhibit Th17 cell–mediated autoimmune diseases, reveal a mechanism by which viliain A acts as an anti-inflammatory therapy in rheumatoid arthritis, and indicate that the enzymatic activity of USP4 could be a target for treatment of RHD.

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Disclosures
The authors have no financial conflicts of interest.

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Supplemental FIGURE 1. USP4 interacts with RORyt in nucleus at its DBD domain. (A) HEK293T cells were transfected with FLAG-RORyt, HA-USP4, after 48h transfected cells were harvested and immunoprecipitated using anti-FLAG or anti-HA. Immunoblots were analyzed using anti-HA or anti-FLAG antibodies. (B) FLAG-RORyt-Jurkat cells were incubated with specific antibodies. Representative confocal microscopy images were visualized for endogenous USP4 (red) and RORyt (green). DAPI was used to visualize the nuclei (blue). (C) HEK293T cells were transfected with FLAG-RORyt, HA-USP4 and the mutant C311A, after 48h transfected cells were harvested and immunoprecipitated using anti-HA. Immunoblots were analyzed using anti-FLAG or anti-HA antibodies. (D) Different truncated RORyt constructs were generated as shown and were co-transfected with HA-USP4 to HEK293T cells. Immunoprecipitated using anti-MYC antibody and USP4 were detected by western blotting. (E) Different truncated USP4 constructs were generated as shown and were co-transfected with MYC-RORyt to HEK293T cells. Immunoprecipitated using anti-HA antibody and RORyt levels were detected by western blotting. Shown are representative findings from at least three experiments.
Supplemental FIGURE 2. 

(A) Human naïve CD4+ T cells were differentiated to Th0, Th1, Th2, Th17. mRNA expression were analyzed by qRT-PCR mRNA expression and was normalized for housekeeping gene GAPDH. (B) HEK293T cells were cotransfected with FLAG-RORγt and various doses of HA-USP4 (WT or C311A). IB were analyzed using anti-FLAG or anti-HA antibodies. (C) HEK293T cells were transfected with FLAG-RORγt, HA-USP4, His-Ubiquitin and treated with 20μM MG132 for 3 hrs before harvested. Pull-down using Ni-NTA-beads, ubiquitinated RORγt was visualized by IB using anti-FLAG antibody. (D) FLAG-Jurkat and FLAG-RORγt-Jurkat were transfected with the IL-17A promoter reporter for 2 days and stimulated with PMA and ionomycin (P+I) overnight with fresh medium, then stimulated with or without USP4 for 8h, then analyzed with luciferase activity and western blotting. (E) FLAG-RORγt-Jurkat T cells were stimulated with PMA and 10ng/ml IL-17A. IB were analyzed using anti-FLAG, anti-USP4 and anti-β-Actin antibodies. (F) The correlation between USP4 or RORγt and IL-17A or IL-17F were analyzed within the RHD patient population. (*, P<0.05; **, P<0.001)