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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 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.

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 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'–ACCAATCCCCTGAAGTCCCTC-3'; IL-17A reverse: 5'-GGGGACAGAGTTATGTTGTTG-3' and IL-17F forward: 5'-CTCCTCCCCTGAAT-TACACT-3'; IL-17F reverse: 5'-ACCCAGACCTFCTCCCAAAGTG-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 × 10⁶ cells were loaded onto SDS-PAGE gels, and 0.5 × 10⁶ cells were used for flow cytometry.

Flow cytometry

Anti–RORγt-PE and anti–IL-17A–PerCP/Cy5.5 were purchased from eBioscience. Data were collected on a FACS LSR II (BD Biosciences) and analyzed using FlowJo software.

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.
naive 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 reporter vector, transfected this vector into FLAG-Jurkat or FLAG-RORγt–Jurkat T cells; under USP4 treatment, there was a decreased level of promoter activity after PMA and ionomycin activation. We performed a similar experiment for USP4i treatment confirmed this phenomenon. These data indicate that USP4 stabilizes RORγt by interaction and deubiquitination of K48-linked polyubiquitination.