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Deubiquitination and stabilization of T-bet by USP10

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ABSTRACT

The T-box transcriptional factor T-bet is crucial in the development, differentiation and function of Th1 cells. It drives Th1 immune response primarily through promoting expression of Th1 hallmark cytokine IFN-γ. Although T-bet was found associated with many immune-mediated diseases such as asthma and systemic sclerosis, little is known about the regulation of T-bet stability and function. Here we identified USP10, a carboxyl-terminal ubiquitin-processing protease, could interact with T-bet in the nucleus. Overexpression of USP10 directly inhibited T-bet ubiquitination and increased the expression of T-bet. We further confirmed Quercetin, a reported inhibitor of T-bet, could target USP10. Quercetin treatment downregulated USP10 and promoted T-bet degradation in a proteasome dependent way. Moreover, we found USP10 expression was upregulated in asthmatic patient PBMC, suggesting USP10 may maintain high level of T-bet and IFN-γ to fight against Th2-dominated inflammation.

1. Introduction

T-bet belongs to the T box family of transcriptional factor, controlling the Th1 genetic program in naïve $CD4^+$ T cells [1]. It expresses in several different cell lineages of the hematopoietic system, including DCs, NK cells, NKT cells, B cells and $CD8^+$T cells, and has important roles in the pathogenesis of several autoimmune diseases [2,3]. In the colitis model, a rapid increase of T-bet is showed in the gut mucosal immune system [4]. T-bet expression in DCs is required for the initiation of autoimmune type I diabetes [5]. $Tbx21^{-/-}$ mice exhibit more severe airway hyperresponsiveness and airway remodeling following allergen sensitization and challenge, similar to human chronic asthma [6–8]. Thus it allows of no delay to focus on T-bet and probe further into its function in different diseases.

T-bet could bind to a highly conserved T-box half-site in the IFN-γ promoter and lead to the methylation of the promoter [9]. C-Abl-mediated tyrosine phosphorylation of the T-bet DNA-binding domain was found regulating $CD4^+$ T-cell differentiation and confirmed in allergic lung inflammation [10]. Lys-313 was lately found as a key site required for T-bet to interact with the IFN-γ gene promoter and to assure phosphorylation at Thr-302, thereby suppressing NFAT1 activity [11]. They also found that T-bet expression is controlled by the ubiquitin-proteasomal degradation pathway on the site of Lys-313 [11]. However, the mechanism underlying T-bet regulation by ubiquitination modification remains unclear.

We investigated the role of deubiquitinases involved in T-bet stability and function. Our findings showed that USP10 could stabilize T-bet via deubiquitination, and enhance the secretion of IFN-γ. USP10 was further identified as a target of Quercetin. This suggested the possible mechanism in which Quercetin treatment could inhibit both T-bet and IFN-γ in Th1 cells. The expression level of USP10 is also associated with inflammation. USP10 was highly upregulated in PBMC from asthmatic patients. Altogether, our study described a positive regulator of T-bet and revealed an unknown mechanism modulating T-bet protein expression level that ultimately affects the inflammatory process, with implications for a variety of diseases resulting from hyper-inflammation responses.

2. Materials and methods

2.1. Reagents

The antibodies we used were as follows: anti-Flag (M2, Sigma), anti-Myc (9E10, Santa Cruz), anti-HA (F-7, Santa Cruz), anti-β-actin (AC-74, Sigma), anti-GAPDH (1C4, Sungene Biotech), anti-T-bet (4B10, eBioscience) and anti-USP10 (2E1, Abcam) CHX and MG132 was purchased from Merck. Protein AG-beads were obtained from

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Santa Cruz. Quercetin dehydrate powder was purchased from Shanghai Sangon Biotech and dissolved in Propylene glycol.

2.2. Plasmids

The Plasmids pIPHA-T-bet, pIPMyc-USP10, pFLAG-Ubi and β-gal were maintained in our lab. USP10 C424A mutant was made using the QuickChange site-directed mutagenesis kit (Stratagene) according to the manufacturer's standard procedure, and confirmed by DNA sequencing. Primers were designed on PrimerX software. OC2-Luc was amplified from Human genomic DNA and cloned into pGL3-Basic reporter vector (Promega). The cloning primers are: 5′-cctccacagccactggtagcttc-3′ and 5′-gtgcccagactttccattgtcagctc-3′.

2.3. Cell culture and transfection

HEK293T cells and Hela cells were cultured in Dulbecco's modified Eagle's medium(DMEM, Hyclone, Thermo) containing 10% fetal bovine serum (FBS), 100 U/ml penicillin and streptomycin at 37 °C in the presence of 5% CO_2. The cells were transfected with the indicated plasmids using Polyethylenimine (PEI, Sigma) according to the manufacturer's instructions.

2.4. Immunoblot analysis and immunoprecipitation

Cells were lysed in RIPA buffer containing 50 mM Tris/HCl, pH 7.4, 1% Nonidet P-40, 0.5% Na-deoxycholate, 150 mM NaCl, 1 mM EDTA, with 1 mM PMSF, 1 mM Na_3VO_4, 1 mM NaF and protease inhibitor (Sigma), followed by immunoprecipitation with the indicated antibodies, separation by SDS/PAGE, and analysis by Western blotting. The blotting exposure was developed with LAS-4000 Mini system. Where applicable, band density indicating protein amount was quantified using Image J software.

2.5. Human $CD4^+$ T cells isolation

Human PBMCs were isolated by Ficoll (GE) from the buffy coat of healthy donors (Shanghai Blood Center). Human $CD4^+$ T cells were purified using a FACS ARIA II cell sorter (BD). The purity of the sorted cells was 95–99%. The purified human $CD4^+$ T cells were expanded with anti-CD3/CD28 beads (Invitrogen) in X-Vivo media (Invitrogen) with 10% AB serum (Gibco).

2.6. Luciferase reporter assay

The OC2 luciferase reporter plasmid was co-transfected with a β-gal or other target plasmids as indicated into 293T. The cells were lysed and analyzed using a luciferase assay normalized to β-gal activity according to the manufacturer's protocol (Beyotime). Results presented are the mean of three separate experiments, and the error bars indicate standard deviations from the mean.

2.7. Quantitative real-time PCR

Total RNA was extracted from $CD4^+$ T cells with TRIzol reagent (Invitrogen) following the manufacturer's instructions. RNA was quantified and complementary DNA was reverse-transcribed using PrimeScript® RT reagent Kit (Takara) following the manufacturer's instructions. PCR reactions for detecting human genes were carried out using SYBR green mix (TAKARA) on ABI Prism 7900 Sequence Detection System. Quantification of relative mRNA expression was determined by the formula 2-ΔCT normalized to GAPDH expression. The primers for real-time PCR were listed as follows: *Tbx21*-forward: 5′-cacctgttgtggtccaagttt-3′ and *Tbx21*-reverse: 5′-tgacaggaatgggaacatcc-3′; *FOXP3*-forward: 5′-tcccagagttcctccacaac-3′ and *FOXP3*-reverse: 5′-attgagtgtccgctgcttct-3′; *GATA3*-forward: 5′-ctcattaagccccaagcgaag-3′ and *GATA3*-reverse: 5′-ttttcggtttctggtctgg-3′; *USP10*-forward: 5′-tgcagagttgctggagaatg-3′ and *USP10*-reverse: 5′-ggccttt gcactttggaata-3′; *IFN-γ*-forward: 5′-aacgagatgacttcgaaaagc-3′ and *IFN-γ*-reverse: 5′-atattgcaggcaggacaacc-3′; *GAPDH*-forward: 5′-gagt caacggatttggtcgt-3′ and *GAPDH*-reverse: 5′-gacaagcttcccgttctcag-3′.

2.8. Immunofluorescence

The transfected cells were fixed in 4% formaldehyde, permeabilized in 0.5% Triton-X 100, blocked, and incubated with indicated antibody. Cell nuclei were stained with DAPI dye. And slides were imaged on a laser microscope (LEICA SP5).

2.9. Statistical analysis

All data are presented as mean ± SEM. Statistical analysis was carried out using Prism software (Graphpad Software). Comparisons between two groups were done by using the Student t test (unpaired, two-tailed). $P < 0.05$ with a 95% confidence interval was considered significant. $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$.

3. Results

3.1. USP10 interacts with T-bet

Several reports have shown that T-bet is regulated by ubiquitination modification and is involved in a proteasome dependent degradation [12,13]. To find out key factors regulating T-bet ubiquitination, we analyzed any possible deubiquitinases (DUBs) interacting T-bet from our pool containing the DUBs highly expressed in T cells. We found the obvious association between T-bet and USP10 in our screening. The physical interaction was demonstrated by endogenous co-immunoprecipitation (Co-IP). USP10 was detected by pull-down with anti-T-bet antibody in human primary $CD4^+$ T cells (Fig. 1A).

Since T-bet is a transcription factor, it mainly localizes in the nucleus, while USP10 is found in both cytoplasm and nucleus [14,15]. We followed up Immunofluorescence after transfecting HA-T-bet and Myc-USP10 into Hela cells and found that the majority of USP10 expressed in the nucleus and colocalized with T-bet (Fig. 1B). The interaction and colocalization suggested USP10 is a binding partner of T-bet.

3.2. USP10 stabilizes T-bet protein via deubiquitination

As a transcription factor, T-bet could bind to the promoter of several genes such as IFN-γ and ONECUT (OC2) and regulate their transcription [16]. To test whether USP10 could affect T-bet function, we constructed a luciferase report gene under the control of OC2 promoter (OC2-Luc). The luciferase activity was enhanced when overexpression of T-bet. The cotransfection of USP10 could significantly upregulated T-bet mediated transcription activation (Fig. 2A), suggesting that USP10 may act as a positive regulator of T-bet.

We further wondered whether USP10 could affect T-bet protein stability. Then we treated cells with cycloheximide (CHX), the protein biosynthesis inhibitor, to measure the half-life of T-bet protein. In the presence of CHX, T-bet was reduced at the protein level; however, the administration of proteasome inhibitor MG132 prevented T-bet degradation in CHX treatment, which is consistent with the previous reports that T-bet undergoes a proteasome dependent pathway (Fig. 2B). When cotransfected with USP10, T-bet degradation by CHX was inhibited (Fig. 2B), demonstrating USP10 stabilized T-bet protein and prolonged the half-life of T-bet.

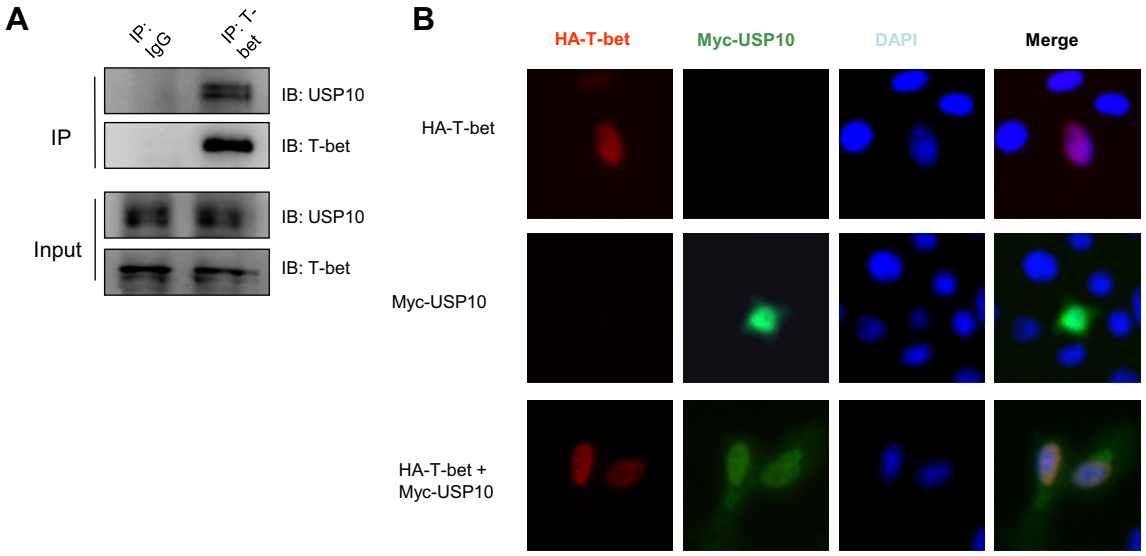

Fig. 1. USP10 interacts with T-bet. (A) $CD4^+$ T cells from PBMC were harvested and lysed with RIPA buffer. The cell lysate was immunoprecipitated with anti-T-bet antibody or mouse IgG control. The results of immunoprecipitation were analyzed by Western blotting. (B) HA-T-bet and Myc-USP10 were transfected into Hela cells. Cell samples were harvested 48 h post transfection and stained with anti-HA antibody followed by Cy3-conjugated anti-rabbit antibody (Red), and anti-Myc antibody followed by 488-labeled anti-mouse antibody (Green) and nuclei stained with DAPI (Blue). The fluorescence signal was detected on a laser confocal microscope. Shown are representative findings from at least three independent experiments.

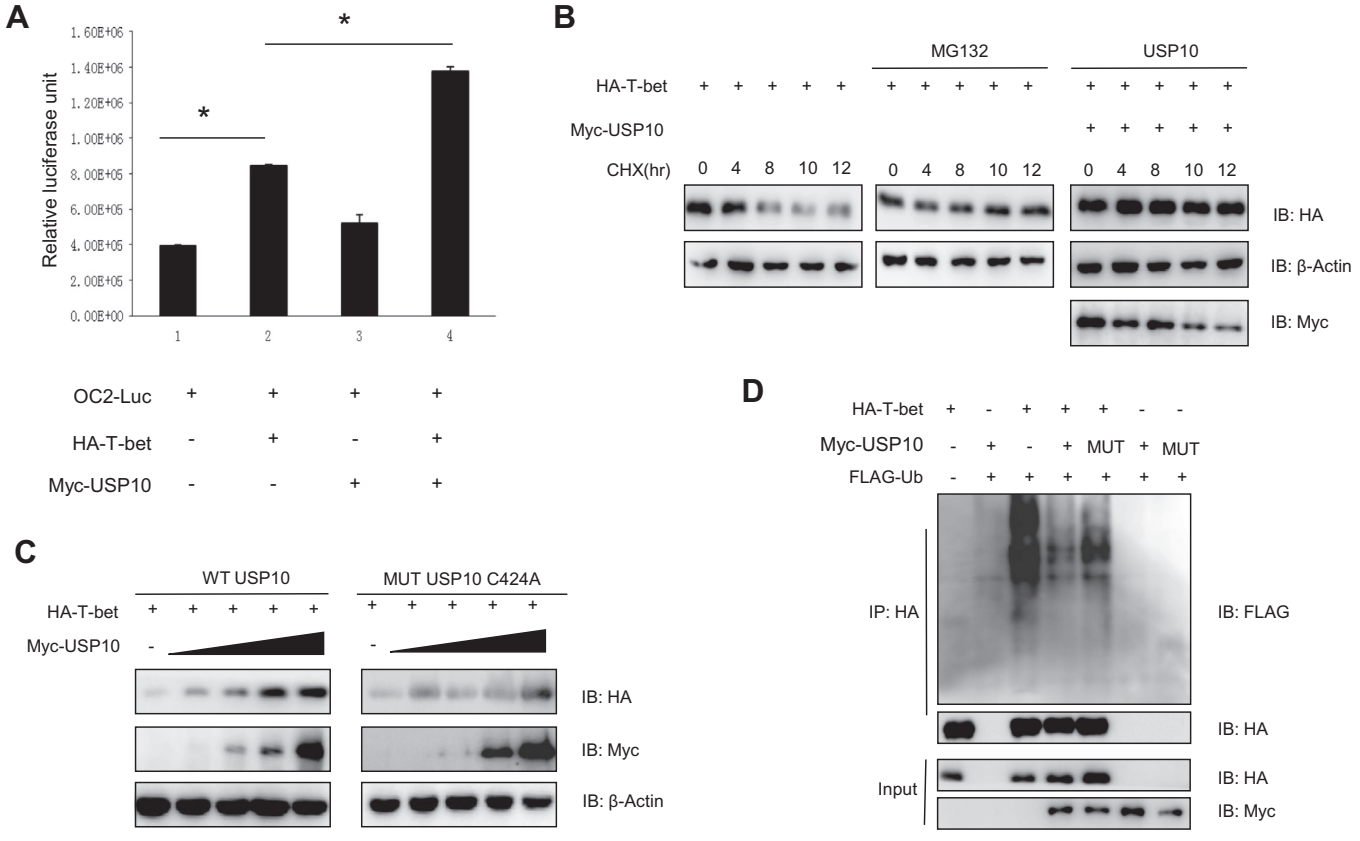

Fig. 2. USP10 stabilizes T-bet protein via deubiquitination. (A) The OC2-promoter luciferase reporter gene was co-transfected with HA-T-bet and/or Myc-USP10 into 293T cells. Luciferase activity was detected by standard methods with β-gal activity serving as a control. The figure is representative of at least 3 independent experiments showing the mean of 3 replicates +/− SEM. (B) HA-T-bet was transfected or co-transfected with Myc-USP10 into 293T cells. Cells were treated with 5 μg/ml CHX with or without the addition of MG132. Cells were harvested at the indicated time-points and analyzed by Western blotting. (C) 293T cells were co-transfected with HA-T-bet and the indicated doses of Myc-USP10 or its mutant plasmid. The cells were harvested 48 h post transfection and Western blotting was analyzed using the indicated antibodies. (D) HA-T-bet, Myc-USP10 or its mutant and FLAG-Ub were co-transfected into 293T. The cell lysate was immunoprecipitated with anti-HA antibody and Western blotting was analyzed using the indicated antibodies.

As a deubiquitinase, USP10 belongs to the ubiquitin-specific protease family of cysteine proteases. Cysteine424 site on USP10 is crucial for its hydrolase activity. We next compared the wild type USP10 and its C424A mutant defective in enzymatic activity. Increasing amount of USP10 was shown to upregulate T-bet expression, but the C424A mutant was unable to enhance the protein level of T-bet (Fig. 2C). Thus, USP10 stabilized T-bet dependent on its hydrolase activity.

We further examined the deubiquitination activity of USP10 on T-bet. HA-T-bet was cotransfected with Myc-USP10 and FLAG-Ub into 293T cells and MG132 was treated before harvesting. The ubiquitinated protein was detected by anti-FLAG antibody after immunoprecipitated by anti-HA antibody. Polyubiquitination of T-bet was dramatically reduced with the addition of USP10, but the C424A mutant failed to remove the ubiquitin chain from T-bet (Fig. 2D), which suggested enzymatic activity of USP10 is required for T-bet deubiquitination. These findings support the notion that USP10 acts as a deubiquitinase of T-bet to stabilize its protein.

3.3. Quercetin targets USP10 for degradation

Previous reports showed a flavonoid compound Quercetin could downregulate IFN-γ in Th1 cells and mediate the degradation of T-bet in a proteasome pathway [12]. We want to know whether Quercetin will modulate USP10. We treated transfected 293T cells with Quercetin. Both T-bet and USP10 decreased with the increasing of Quercetin, but the level of GATA-3 had no change in the presence of Quercetin (Fig. 3A). In Human $CD4^+$ T cells, Quercetin treatment also decreased endogenous T-bet and USP10 expression but not GATA-3 (Fig. 3A). Therefore, USP10 may be also a target of Quercetin.

USP10 could prevent T-bet degradation and enhanced its protein level. The enhancement of T-bet was inhibited under Quercetin treatment because of the degradation of USP10 by Quercetin (Fig. 3B). Thus, Quercetin repressed USP10 mediated T-bet stabilization. When treated with MG132, T-bet degradation by Quercetin was inhibited, which is consistent with the previous report. However, Quercetin could also target USP10 for degradation in a proteasome pathway, but not affect GATA-3 level (Fig. 3C). These findings suggested the mechanism by which T-bet was degraded by the proteasome under Quercetin treatment. Quercetin may target USP10 for degradation and then lead to the destabilization of T-bet.

3.4. USP10 expression is associated with inflammation

Finally, we investigated the importance of USP10 in human diseases. By promoting IFN-γ secretion and inducing Th1 cell migration, T-bet controls many aspects of inflammatory diseases. We analyzed gene expression levels in the PBMC from allergic asthma patients and healthy donors. The mRNA levels of $CD4^+$ T cell transcription factors including T-bet, GATA3, and FOXP3 were all increased in asthmatic patients. Consistently, IFN-γ was also highly upregulated in the patients. When we examined the expression level of USP10, we found asthma patients had enhanced expression of USP10 (Fig. 4). Thus, USP10 could be upregulated in the inflam-

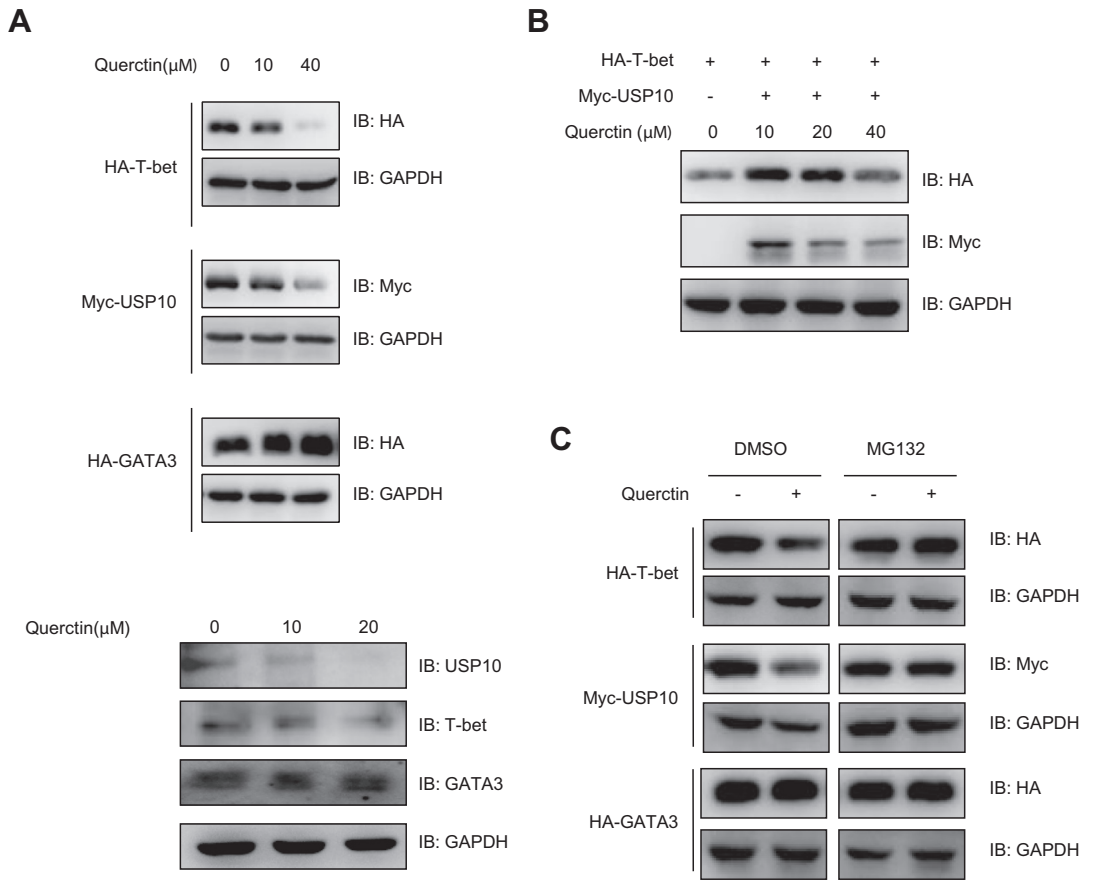

Fig. 3. Quercetin targets USP10 for degradation. (A) 293T cells transfected with HA-T-bet, Myc-USP10 or HA-GATA3 were treated with Quercetin at different concentration for 8 h (Up). The primary $CD4^+$ T cells were treated with Quercetin at different concentration for 8 h (Bottom). The cell lysate was analyzed with Western blotting. (B) HA-T-bet and Myc-USP10 were transfected into 293T cells. The cells were treated with Quercetin at different concentration for 8 h before harvesting. The cell lysate was analyzed with Western blotting. (C) 293T cells transfected with HA-T-bet, Myc-USP10 or HA-GATA3 were treated with 40 μM Quercetin for 8 h with or without MG132 addition. The cell lysate was analyzed with Western blotting.

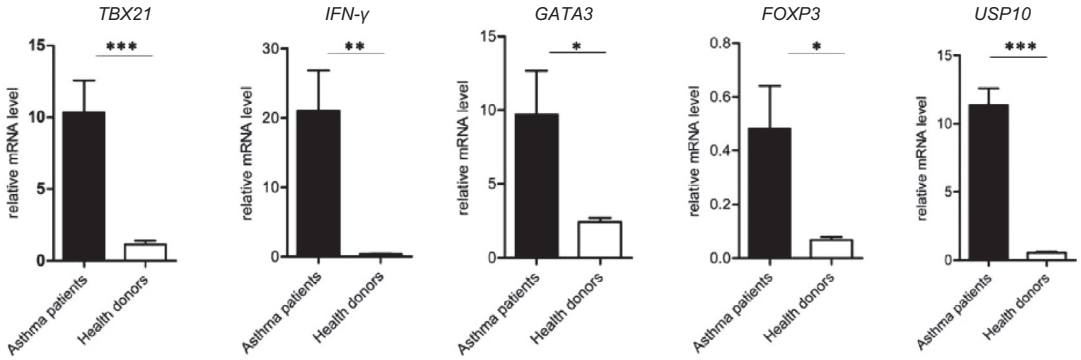

Fig. 4. USP10 expression is associated with inflammation. PBMCs were isolated from asthma patients and healthy donors. mRNA from samples were prepared for the detection of *TBX21*, *IFN-γ*, *GATA3*, *FOXP3* and *USP10* expression level by qPCR.

mation conditions, suggesting that USP10 could be a target of diagnostics and therapy in inflammatory diseases.

4. Discussion

T-bet is the major T-box transcription factor of Th1, promoting Th1 development [1]. In previous study, more attention was paid on the regulation of transcriptional level of T-bet, less in post-translation. Our results show that USP10 interacts with T-bet and mediates the deubiquitination of T-bet, stabilizes and increases the expression of T-bet. Our study provides a new mechanism by which T-bet protein is stabilized and effect on Th cell differentiation.

USP10 expresses in many mammalian cells. The mechanism that USP10 can enhance T-bet expression and deubiquitinate T-bet to make it stable is quite meaningful in the Th cell differentiation and balance. We also confirmed a significant increase of USP10 in the PBMCs from patients with inflammation. So under the inflammatory condition, the level of USP10 might change along with the severity of inflammation and regulate it by deubiquitinating T-bet.

Quercetin (3,3′,4′,5,7-pentahydroxyflavone dihydrate), a flavonoid compound biosynthesized by plants, can modulate a variety of inflammatory responses [12]. In vivo mice model studies showed that Quercetin could down regulate the allergic airway inflammation via oral or intraperitoneal routes or by aerosol [13]. In vitro studies carried out that Quercetin possess the antioxidant and anti-inflammatory property, blocking some inflammatory mediators, adhesion as well as adhesion molecules expression, inducible enzymes and nuclear transcription factor activation [17]. One study found that Quercetin could suppress the protein level of T-bet in T helper cells and downregulate IFN-γ [12]. But later on, another group found Quercetin could reduce Th2 cytokine production but increase Th1 cytokine in OVA-sensitized and challenged mice model [13]. Our data explored the exact signaling pathway about the Quercetin-mediated T-bet reduction in immune response. In Quercetin treated human $CD4^+$ T cells, both T-bet and USP10 expression decreased along with the increasing of Quercetin while GATA-3 protein expression seemed had no significant change. This pathway could be the reason of T-bet protein level reduction after Quercetin treatment.

T-bet demonstrates complex role in the immune response and is involved in many inflammatory diseases. T-bet-deficient mice show greater resistance to the development of several inflammatory and autoimmune diseases, including inflammatory bowel disease [4], experimental autoimmune encephalomyelitis [18,19], arthritis [20], systemic lupus erythematosus [21] and type 1 diabetes [5,7]. After continuous allergen exposure, T-bet is increased in inflammatory tissue, modulating Th1/Th2 balance and regulating airway inflammatory response [7]. We tested the mRNA level of *TBX21*, *IFN-γ*, *GATA3*, *FOXP3* and *USP10* in both allergic asthma patients and healthy donors. These five genes were shown to be increased in asthma patients (Fig. 4).

Our results revealed that USP10 could interact with T-bet and stabilize it via deubiquitination to inhibit its degradation by the proteosome. The upregulation of USP10 maintained T-bet in a steady state under inflammation. Quercetin was confirmed to be the exact compound to downregulate USP10 and T-bet. We believe that the USP10-dependent T-bet deubiquitination and stabilization can regulate antigen induced immune disorder especially in Th1-specific inflammation. Thus, appropriate decreasing USP10 level may contribute to the T-bet degradation and inflammation attenuation. Quercetin was shown as a potential anti-inflammatory drug in autoimmune disease.

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