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Identification of the E3 Deubiquitinase Ubiquitin-specific Peptidase 21 (USP21) as a Positive Regulator of the Transcription Factor GATA3

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Background: GATA3 is regulated both transcriptionally and post-translationally. GATA3 is important for the function of FOXP3+ Treg cells. The expression of the transcription factor GATA3 in FOXP3+ regulatory T (Treg) cells is crucial for their physiological function in limiting inflammatory responses. Although other studies have shown how T cell receptor (TcR) signals induce the up-regulation of GATA3 expression in Treg cells, the underlying mechanism that maintains GATA3 expression in Treg cells remains unclear. Here, we show how USP21 interacts with and stabilizes GATA3 by mediating its deubiquitination. In a T cell line model, we found that TcR stimulation promoted USP21 expression, which was further up-regulated in the presence of FOXP3. The USP21 mutant C221A reduced its capacity to stabilize GATA3 expression, and its knockdown led to the down-regulation of GATA3 protein expression in Treg cells. Furthermore, we found that FOXP3 could directly bind to the USP21 gene promoter and activated its transcription upon TcR stimulation. Finally, USP21, GATA3, and FOXP3 were found up-regulated in Treg cells that were isolated from asthmatic subjects. In summary, we have identified a USP21-mediated pathway that promotes GATA3 stabilization and expression at the post-translational level. We propose that this pathway forms an important signaling loop that stabilizes the expression of GATA3 in Treg cells.

Results: USP21 prevents the ubiquitination and degradation of GATA3. USP21 is a positive regulator of GATA3 expression. The identification of a molecular pathway where USP21 positively controls GATA3 expression at the post-translational level reveals USP21 as a potential drug target to manipulate the function of T cells.

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3 The abbreviations used are: nTreg, naturally occurring regulatory T cell(s); TcR, T cell receptor; Teff, T effector; USP15, ubiquitin-specific peptidase 15; PBMC, peripheral blood mononuclear cells(s); qPCR, quantitative PCR; F, forward; R, reverse; GATA3, JBC binding protein 3.

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USP21 Is a Positive Regulator of GATA3 Expression

expression during Th2 polarization, the expression of GATA3 in nTreg cells is independent of STAT6 signaling (5). Although GATA3 has been shown to inhibit TGF-β1-dependent FOXP3 up-regulation during the polarization of induced Treg cells (10), GATA3 and FOXP3 are highly expressed in nTreg cells, and GATA3 expression has been shown to be critical for nTreg cell function. The deficiency of GATA3 specifically in FOXP3+ Treg cells results in the development of spontaneous inflammatory disorders in mice. Furthermore, GATA3-null Treg cells are defective in their immune-suppressive function both in vitro and in vivo (13). GATA3 has also been reported to be pivotal to Treg cell physiology during inflammation, for maintaining the capacity of Treg cells to accumulate at inflamed sites and the sustaining of high levels of FOXP3 expression (14). However, the underlying mechanisms that allows for the elevated and maintained expression of GATA3 in FOXP3+ Treg cells remains unclear.

Our previous work has indicated that FOXP3 is a target for post-translational modification by acetylation, phosphorylation, and ubiquitination (15, 16). We hypothesize that the up-regulation of GATA3 in FOXP3+ Treg cells arises due to its post-translational modification through deubiquitination. Ubiquitination can rapidly direct proteins toward degradation. Protein ubiquitination involves various cellular processes and its involvement in immune signal transduction pathways has been extensively studied (17, 18). The deubiquitinase family is currently comprised of ~100 members in mammalian cells that oppose the function of E3 ubiquitin ligases (19). For example, deubiquitinases such as ubiquitin thioesterase CYLD, A20, Cezanne, ubiquitin-specific peptidase 15, USP21, and USP31 participate in the regulation of the NF-κB pathway (20–25). Other deubiquitinases, including USP7 and USP10, are involved in regulating p53 localization and function (26, 27).

Here, we show that the expression of the E3 deubiquitinase USP21 is positively regulated by TcR signals and can be further up-regulated by FOXP3. We also reveal how USP21 stabilizes GATA3 expression at the post-translational level. Through screening for the putative regulators of GATA3, we found that the overexpression of USP21 stabilized and increased GATA3 expression. We also found that the overexpression of wild-type USP21 but not its enzymatic-inactive mutant C221A specifically promoted the up-regulation of GATA3 expression. This suggests that the deubiquitinase activity of USP21 is essential for stabilizing GATA3. By co-immunoprecipitation, USP21 was found to interact with GATA3. Moreover, we found that T cell activation via CD3/CD28 co-stimulation up-regulated USP21 and GATA3 expression. Interestingly, USP21 expression was potentiated in the presence of FOXP3. shRNA-mediated knockdown of USP21 down-regulated GATA3 expression in primary Treg cells and decreased the expression of GATA3 target genes in Th2 cells. Thus, the mechanism by which USP21 stabilizes GATA3 is through the prevention of GATA3 degradation by facilitating its deubiquitination. Finally, the overexpression of wild-type USP21 promoted GATA3-mediated expression of a luciferase reporter downstream from the Il4 promoter. Thus, we have unveiled a molecular pathway that controls the expression and activity of GATA3 at the post-translational level, which suggests a role for USP21 in the differentiation and function of T cells.

EXPERIMENTAL PROCEDURES

Cell Culture and Transfection—HEK 293T cells and U2OS cells were cultured in DMEM containing 10% fetal bovine serum (FBS) and transfected using Lipofectamine 2000 (Invitrogen) according to the manufacturer’s instructions. Immunoprecipitation and Immunoblotting—Cells were washed with pre-chilled phosphate-buffered saline (PBS) and lysed in radioimmune precipitation assay buffer consisting of 20 mM Tris–HCl, 135 mM NaCl, 1 mM EDTA, 1% Nonidet P-40, 10% glycerol, 1% protease inhibitor mixture (Sigma), and 1 mM PMSF. These cell lysates were used for immunoprecipitation and/or immunoblotting. Antibodies against FLAG and β-actin were purchased from Sigma; anti-HA antibody (F-7) was purchased from Santa Cruz Biotechnology. Anti-Myc (22E8) antibody was purchased from Sungene Biotech. Anti-CD4-FITC (RPA-T4), anti-CD25-PE (BC96), and anti-CD127-APC (BC96) were purchased from Biologend.

Immunostaining—U2OS Cells were fixed in 2% PFA and permeabilized with 1% Triton. Anti-USP21 rabbit polyclonal antibody (AP069a, Abgent) and anti-GATA3 (HG3-31, sc-268) monoclonal antibody were used to stain for the overexpressed myc-USP21 and FLAG-GATA3. Cells were then stained with DAPI to show the nuclei.

Treg and T Cell Isolation and Expansion—CD4+CD25−CD127lo Treg and CD4+CD25− T eff T cells were isolated from peripheral blood mononuclear cells (PBMC) by FACS on a BD FACS ARIA II sorter. Primary cells were expanded using anti-CD3/CD28 DynaBeads (Invitrogen) at a cell to bead ratio of 1:3 in X-VIVO media (Lonz) supplemented with 10% human AB serum, 1% GlutaMax (Invitrogen), 1% sodium pyruvate (Invitrogen), and 1% penicillin/streptomycin (Invitrogen). Treg cells were expanded in the presence of a high concentration of IL-2 (500 units/ml) and rapamycin (100 nM) for 10 days followed by resting in a lower concentration of rIL-2 (100 units/ml). Teff cells were expanded with no addition of other factors for 10 days before analysis. Th1 cells were polarized with rIL-12 (1 ng/ml) and anti-IL-4 (10 μg/ml) antibody, Th2 cells were polarized with rIL-4 (20 ng/ml) and anti-IFNγ (10 μg/ml) antibody, and iTreg cells were polarized with 10 ng/ml TGF-β, 100 ng/ml All-trans retinoic acid (atRA) and 100 units/ml rIL-2, in primary cell cultures containing anti-CD3/28 Dynabeads (Invitrogen).

T Cell Culture and Activation—Jurkat T cells were cultured in RPMI 1640 medium containing 10% FBS, 1% sodium pyruvate and 1% non-essential amino acids. For SZ-4 cells, the medium was supplemented with 100 units/ml recombinant human IL-2 (R&D Systems). T cells were activated using soluble antibodies against CD3 (1 μg/ml, Hit3a, Biologend) and CD28 (2 μg/ml, CD28.2, Biologend) for long term culture or treated with phorbol 12-myristate 13-acetate (PMA) (50 ng/ml) plus ionomycin (1 μM).

Luciferase Reporter Assay—HEK-293T cells or Jurkat T cells were transiently co-transfected with firefly luciferase reporter vectors, effector vectors, and the Renilla luciferase vector. After 48 h, cells were harvested in passive lysis buffer, and luciferase...
assays were performed using the Dual-Luciferase reporter assay system (Promega).

**Lentiviral Constructs and Infection**—The shRNA lentiviral vectors pLKO.1 shUSP21-1/2 or pLKO.1 shCK were transfected into the HEK 293T cells with the lentivirus packing vector Delta 8.9 and VSVG envelope glycoprotein. The pLKO.1 vector either contained GFP or the gene that provided cells with resistance to puromycin. Viral supernatants were harvested after 48 h. Primary human Th2 cells were expanded as detailed above and transduced with virus along with a secondary anti-CD3/28 stimulus (four cells to one bead). nTreg cells were transduced similarly after their expansion. The following shRNA sequences were used in this study: 5'-CCACTTTT-GAGACGTAGCACCCTCT-C3' (shUSP21-1) and 5'-GACCCCTCT-GCAATATCCTTTC3' (shUSP21-2).

**ChIP on Quantitative PCR (qPCR)**—2 × 10^6 Jurkat T cells, which stably expressed HA-FOXP3 were activated by antibodies against CD3 (1 μg/ml) and CD28 (2 μg/ml). Cells were then cross-linked with formaldehyde, and the chromatin sonicated into 500–700-bp fragments. The sheared chromatin was immunoprecipitated with anti-HA antibody (sc-7392, Santa Cruz Biotechnology) or mouse anti-IgG only as a negative control. The pulled down DNA fragments were subjected to qPCR analysis. The human USP21 promoter primers used for the qPCR experiments were as follows: Probe 1-F, 5'-CTGAAACCTTGACATGATGAC-3'; Probe 1-R, 5'-CAAAGCATG-GATAATATCACTTT-C3'; Probe 2-F, 5'-CTTTTTGCCGTTTCTGGTCTGG-3'; Probe 2-R, 5'-GGCTGCAAATGAGCAGCACTTCC-3'.

**Asthma Patients and PBMC Isolation**—The population consisted of patients with asthma aged between 16 to 60 years from the asthmatic clinic of Ruijin Hospital (Shanghai, China). The diagnosis of asthma was made by the allergy and pulmonary physicians staffing the tertiary care clinics and was based on their established criteria. Subjects had received a physical examination, asthma control assessment, an administered skin prick test of allergen extracts, and spirometry to test lung function. The study was approved by the ethics committee of the hospital. PBMC were isolated by Ficoll-density gradient centrifugation and then stained with the fluorescently labeled antibodies CD4-FITC, CD25-PerCP-cy5.5, and CD127-PE to sort for Treg cells on a FACS ARIAII cell sorter. Total RNA was extracted from human PBMC using TRIzol reagent (Invitrogen). cDNA was synthesized using a reverse transcriptase kit (TaKaRa), followed by quantitative qPCR analysis (SYBR Green; Applied Biosystems). The data were analyzed, and graphs were generated on SPSS software (IBM). Primers used for the qPCR of USP21, GATA3, and FOXP3 mRNA were as follows: FOXP3-F, 5'-CCCCAGATGTCCTCCACCAAC-3' and FOXP3-R, 5'-ATGAGTGTGCCGTCTCTCT-3'; GATA3-F, 5'-CTGATGAGAACCCAGCGGAG-3' and GATA3-R, 5'-TTTTGGCTTTCTGTCGTGG-3'; USP21-F, 5'-ATCCGGATGCTCTTCCAGA-G3' and USP21-R, 5'-CATTAGGTTGGCGTGATCAT-3'; β-actin-F, 5'-GGACCTCAGAGCAGAA-GATGG-3' and β-actin-R, 5'-AGCCTGTGTTGGGCCTACAG-3'; and GAPDH-F, 5'-GAGTCACGGATTTGGTCGT-3' and GAPDH-R, 5'-GACAAGCTTCCGTTTCAG-3'.

**RESULTS**

**USP21 Up-regulates GATA3-mediated IL4 and IL5 Activity**—FOXP3-expressing T cells have been reported to express GATA3 after TcR stimulation (5, 28). To explore whether any potential deubiquitinases contribute to GATA3 stabilization in FOXP3-expressing cells, we first scanned through the available online resources (e.g. the BioGPS website and Ref. 29) and cloned 16 deubiquitinases that were found highly expressed in T cells. We then tested their effect on GATA3-mediated activation of the Il4 and Il5 promoters by the co-transfection of each individual deubiquitinases together with GATA3 and the luciferase reporter constructs containing either the Il4 (Fig. 1A) or Il5 (Fig. 1B) promoters into HEK 293T cells. USP21 significantly up-regulated GATA3 mediated luciferase activity under the control of both the Il4 and Il5 promoters. This positive regulation of GATA3 activity by USP21 was further enhanced after PMA/ionomycin stimulation in Jurkat T cells (Fig. 1, C and D).

**USP21 Interacts with and Stabilizes GATA3 Expression**—Expression vectors containing FLAG-tagged GATA3 (FLAG-GATA3) and Myc-tagged USP21 (Myc-USP21) were co-transfected and ectopically expressed in HEK 293T cells followed by reciprocal co-immunoprecipitation to examine whether USP21 could interact with GATA3 to promote its function. We found a positive interaction between USP21 and GATA3 when GATA3 or USP21 were co-immunoprecipitated (Fig. 2A). Because USP21 is an ubiquitin-specific protease, it is possible that USP21 stabilizes GATA3 by preventing its ubiquitination and degradation. We observed that the increase in GATA3 expression positively correlated with the increase in dosage of the co-expressed USP21 construct, whereas the USP21 deubiquitinase-deficient (C221A) mutant could not facilitate the stabilization of GATA3 levels (Fig. 2B). To confirm these results, we next treated cells transfected with or without USP21 with the protein synthesis inhibitor cycloheximide for the duration of the indicated time points. We found that the overexpression of USP21 effectively rescued GATA3 from its degradation (Fig. 2C).

**USP21 Promotes the Deubiquitination of GATA3**—Because USP21 is an ubiquitin-specific protease and was found to stabilize GATA3 expression, we reasoned that the function of USP21 toward GATA3 was to oppose the function of E3 ubiquitin ligases that promote its degradation. To test this, we co-transfected FLAG-GATA3 and HA-ubiquitin into HEK 293T cells, with or without Myc-USP21; after 2 days, these cells were treated with the proteasome inhibitor MG132 for 4 h before they were lysed for immunoprecipitation analysis. Immunoprecipitation was carried out using anti-FLAG antibody and then analyzed by immunoblotting with anti-HA antibody and reprobed with anti-FLAG antibody. As shown in Fig. 2D, we observed the accumulation of ubiquitinatated GATA3 after the inhibition of the proteasome by MG132. However, the overexpression of USP21 significantly decreased GATA3 ubiquitination, which suggests that USP21 may act as a deubiquitinase of GATA3. We next sought to identify the localization of USP21 and GATA3 by immunofluorescence. Myc-USP21 and FLAG-GATA3 were co-transfected into U2OS cells and stained with
antibodies against USP21 and GATA3 (Fig. 2E). USP21 was found localized both in the nucleus and cytoplasm, whereas GATA3 mainly colocalized with USP21 in the nucleus. This suggests that USP21 colocalizes with GATA3, which could contribute to the stabilization of GATA3.

**TcR Signaling Induces USP21 and GATA3 Expression and Is Potentiated by FOXP3**—Previous studies have shown that Treg cells up-regulate the expression of GATA3 after TcR stimulation (5). Therefore, we decided to test whether USP21 could be induced after TcR stimulation. We treated control (HA-expressing) with anti-CD3/CD28 antibodies. After 48 h of stimulation, endogenous USP21 and GATA3 were detected (Fig. 3A). However, in the presence of FOXP3 (HA-FOXP3) Jurkat T cells stably express human FOXP3) USP21 and GATA3 expression was further potentiated (Fig. 3A). This data suggests that FOXP3 may partake in the induction and maintenance of USP21 and GATA3 expression in FOXP3+ T cells. To further confirm that USP21 regulates the expression of GATA3, we performed knockdown experiments of USP21 in primary Treg cells via lentiviral transduction of shRNA targeting USP21. To enrich cells that expressed the shRNA, the cells were selected using puromycin. These cells were then tested for their expression of USP21, GATA3, and FOXP3 by Western blotting. We found that GATA3 and FOXP3 protein levels were down-regulated in the USP21 knockdown cells (Fig. 3B). We then used qPCR and flow cytometry to test the effect of USP21 depletion on GATA3 expression of GFP sorted cells. (GFP is encoded by the lentiviral vector.) The GFP-sorted cells were first tested for the expression of USP21, GATA3, and FOXP3 by qPCR. Here, we found that USP21 was effectively depleted, accompanied by the average reduction in GATA3 and FOXP3 that was not statistically significant (Fig. 3C). By flow cytometry, we found a significant reduction in GATA3 levels (Fig. 3D, left). Moreover, the reduction in Median Fluorescence Intensity (MFI) levels of GATA3 in the USP21 depleted cells relative to the control shRNA transduced cells showed statistical significance (Fig. 3D, right). Next, we tested the expression of USP21 in Th1, Th2, and iTreg cells (Fig. 3E). Here, we found that USP21 was highly up-regulated in both Treg and Th2 cells. We then examined the effect of USP21 depletion in Th2 cells and found that upon the depletion of USP21, the expression of the GATA3 target genes IL4 and IL13 were significantly reduced (Fig. 3F). These results suggest that USP21 may control GATA3 expression and function in both Treg and Th2 cells.

**FOXP3 Binds to the USP21 Promoter and Promotes USP21 Transcription after TCR Stimulation**—Because USP21 expression is up-regulated in FOXP3 expressing Treg cells, we decided to test whether FOXP3 could bind to the USP21 pro-
moter to directly regulate the transcription of USP21. We analyzed the DNA sequence of the human USP21 promoter and found a potential FOXP3 binding site within the -352 and -346 region (Fig. 4A). The USP21 promoter was cloned into a luciferase reporter and tested for the effect of FOXP3 on the promoter. The overexpression of FOXP3 could effectively activate the USP21 promoter as shown by the increase in luciferase activity in a dose-dependent manner. Moreover, FOXP3-induced luciferase activity was blocked following a mutation of the FOXP3 binding site within the USP21 promoter (Fig. 4B).

To demonstrate the direct binding of FOXP3 to the USP21 promoter, we performed chromatin immunoprecipitation (ChIP) followed by qPCR in HA-FOXP3 Jurkat T cells. After CD3/CD28 stimulation for various durations, we found that the transient recruitment of FOXP3 to the USP21 promoter was dramatically increased at 30 min after stimulation (probe 2, comparing with probe 1 in Fig. 4C). These data suggest that FOXP3 specifically binds to the USP21 promoter after TcR stimulation.

FOXP3, GATA3, and USP21 Forms a Positive Feedback Loop—Previous studies have reported that murine FoxP3 interacts with GATA3 (11, 30). Here, we confirmed the interaction between human FOXP3 and GATA3 by co-immunoprecipitation (Fig. 5A). Previous studies have also demonstrated that the expression of GATA3 in FOXP3/nTreg cells is essential for Treg cell-mediated control of immune homeostasis (5, 31). We analyzed the mRNA expression of FOXP3, GATA3, and USP21 in nTreg and Teff cells isolated from human PBMC by qPCR and found that both GATA3 and USP21 mRNA were highly expressed in FOXP3/nTreg cells (Fig. 5B). This result supports the notion that USP21 may regulate GATA3 protein level in Treg cells. We then decided to test whether the expression of FOXP3, GATA3, and USP21 form a positive feedback loop, i.e. FOXP3 activates USP21 transcription, USP21 then stabilizes GATA3, and GATA3 in turn promotes FOXP3 function. To test our hypothesis, we transfected USP21 and FOXP3 at increasing doses, with or without GATA3, into HEK 293T cells and tested the expression of GATA3 and FOXP3. As shown in
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Fig. 5, the increase in FOXP3 expression correlated with the increased dosage of USP21 only in the presence of GATA3.

USP21, GATA3, and FOXP3 Are Up-regulated in the Treg Cells of Asthma Patients—It has been reported that Treg cells play a suppressive function in controlling effector lymphocyte activity in the lungs of patients suffering from asthma. Treg cell numbers increase in patients after glucocorticoid treatment (32). We decided to detect the expression level of FOXP3, GATA3, and USP21 in the Treg of asthma patients. PBMC were isolated from the peripheral blood of asthma patients; healthy donor PBMC was used as a control. Treg cells were sorted via FACS by gating for CD4^+/CD25^+/CD127^low^ T cells. Total RNA was then extracted and subjected to qPCR analysis for the detection of USP21, GATA3, and FOXP3 expression. Our data shows how the expression of USP21, GATA3, and FOXP3 were significantly increased in the Treg cells of asthma patients, compared with those from the healthy donor with statistical significance (Fig. 5D). This correlation suggests a combined role of FOXP3, GATA3, and USP21 in the regulation of immune responses during inflammation (Fig. 5E).


FIGURE 4. FOXP3 binds to the USP21 promoter and activates USP21 transcription during TcR stimulation. A, a map of the USP21 promoter and the prediction of putative transcription factor binding sites. B, HEK293T cells were transfected with a vector containing FOXP3 or a control empty vector, plus the USP21-basic promoter luciferase (Luc) reporter with and without a mutation (mut) in the putative FOXP3 binding site, and control murine sarcoma virus (MSV) promoter-β-gal and then analyzed by means of a luciferase assay normalized to β-gal activity. Error bars show the S.D. from the mean of three separate experiments. C, ChiP on qPCR assay. HA-FOXP3 Jurkat T cells were treated with anti-CD3 (1 μg/ml) plus anti-CD28 (2 μg/ml) for various durations. After sonication, cells were then immunoprecipitated using anti-HA antibody. Normal anti-IgG was used as a control. Error bars show the S.D. from the mean in one experiment representative of three independent experiments.

DISCUSSION

GATA3 is an essential and sufficient transcription factor for the polarization and function of the Th2 cell lineage. The expression level and activity of GATA3 in Treg cells must be tightly regulated, as TcR stimulation promotes GATA3 expression without the induction of the expression of Th2-associated cytokines. Although GATA3 has been reported to be constitutively expressed in Treg cells, its expression is independent of IL-4/STAT6 signals (14). The underlying mechanism of how GATA3 expression is maintained in Treg cells remains unclear.

By using a candidate-based screening approach, we have identified that the deubiquitinase USP21 can interact with GATA3 to promote its stability via deubiquitination. Using a luciferase reporter assay, we found that both USP7 and USP21 increased the transcription from the Il4 promoter, whereas USP3 and USP21 showed a similar activity in promoting transcription from the Il5 promoter. We have also shown how USP21 expression is increased upon TcR stimulation and further potentiated in a FOXP3-dependent manner and that USP21 can interact with and stabilize GATA3 expression. After cycloheximide treatment (to inhibit protein synthesis) for 1, 3, and 6 h, USP21 rescued GATA3 from its degradation compared with the control group in which GATA3 was effectively degraded after 6 h. Furthermore, the USP21 mutant C221A lacked the ability to increase GATA3 expression, but we did observe a slight increase in its expression. USP21 may contain other important residues that are important for its deubiquitinase activity or perhaps other redundant pathways may stabilize GATA3 expression; in any case, we hope to explore these possibilities in our future studies. Because USP21 is a ubiquitin-
specific enzyme and can stabilize GATA3, it was likely that USP21 could deubiquitinate the ubiquitin chains on GATA3. Our results show how the overexpression of USP21 dramatically decreases the degree of GATA3 ubiquitination.

We constructed the Jurkat-HA-FOXP3 stable cell line as a Treg cell model to test the regulation of FOXP3 at the post-translational level, bypassing the transcriptional regulation of the FOXP3 gene promoter. After TcR stimulation, we observed the increase in USP21 expression as early as 4 h post-stimulation. However, FOXP3 expression was essential for the further up-regulation of USP21. Interestingly, we observed that the molecular weight of FOXP3 increased after TcR stimulation. We propose that TcR signaling may lead to the post-translational modification of FOXP3, which in turn promotes USP21 transcription. By qPCR, we found that USP21 was highly expressed in primary Treg and Th2 cells.

To further define the role of USP21 in regulating GATA3 expression, we carried knockdown experiments of USP21 in primary Treg and Th2 cells and found that the knockdown of USP21 resulted in the down-regulation of GATA3 protein levels. Th2/GATA3 target genes were also down-regulated in their expression. During the preparation of this manuscript, it was proposed that in mice, GATA3 and FOXP3 form a positive regulatory loop in Treg cells such that FOXP3 and GATA3 bind to the GATA3 and FOXP3 gene regions, respectively, to regulate their expression (33). Here, we found that upon the depletion of USP21 both GATA3 and FOXP3 protein levels were down-regulated; although the average mRNA levels of GATA3 and FOXP3 reduced, it was not at a significant level. However, we do not exclude the possibility that FOXP3 regulates GATA3 in human Treg cells at the gene level as we did observe the reduction of GATA3 transcripts in some instances. It would therefore be interesting to decipher whether there are certain conditions that favor the regulation of GATA3 expression either at the gene or protein level by FOXP3.

The results above suggested that FOXP3 could regulate USP21 transcription. To prove that FOXP3 could bind to the USP21 promoter and activate USP21 transcription, we scanned the USP21 promoter and found a FOXP3 binding motif within the −352 to −346 region. We then carried out ChIP analysis...
and verified that FOXP3 could bind to this region. Our data support a positive feedback loop among USP21, GATA3, and FOXP3, such that, after TCR stimulation, FOXP3 up-regulates USP21 transcription. USP21 interacts and stabilizes GATA3. USP21 and GATA3 then act to up-regulate FOXP3 expression (Fig. 5D). We also speculate that GATA3 may recruit USP21 to the FOXP3 complex to prevent FOXP3 degradation. This working model could explain how GATA3 and USP21 are essential for the self-regulation of FOXP3 in Treg cell function and stability (34, 35). Future studies revealing how this positive feedback loop is controlled, especially during inflammation, are required to precisely understand how FOXP3–Treg cell stability and plasticity are tightly regulated under physiological conditions.

It has been reported that Treg cells play a suppressive role in controlling effector lymphocyte activity in the lungs of patients suffering from asthma. Our data indicates that GATA3, USP21, and FOXP3 are all up-regulated in the Treg cells of asthma patients, which correlates with our finding that implicates a role of USP21 in regulating the immune system. Further studies on different T cell subsets, especially on functional and tissue specific Treg cell subsets of asthma patients would be meaningful to reveal the functional consequences of this co-up-regulation of GATA3, USP21, and FOXP3. Although our present studies mainly focus on FOXP3 + Treg cells, it is equally important to further investigate the molecular mechanism underlying the up-regulation of USP21 in Th2 cells, as GATA3 is crucial for Th2 cell differentiation and function.

In summary, our results provide evidence that USP21 interacts with GATA3 and acts as a GATA3 deubiquitinase. This mechanism may contribute toward the up-regulation of GATA3 expression in Treg cells, which is independent of STAT6 but can be potentiated in a FOXP3-dependent manner. Finally, in Treg cells, USP21, GATA3, and FOXP3 may form a positive loop to promote FOXP3 expression and thus modulate Treg cell activity.

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