Negative regulation of IL-17-mediated signaling and inflammation by the ubiquitin-specific protease USP25

Bo Zhong, Xikui Liu, Xiaohu Wang, Seon Hee Chang, Xindong Liu, Aibo Wang, Joseph M Reynolds & Chen Dong

Interleukin 17 (IL-17) is important in infection and autoimmunity; how it signals remains poorly understood. In this study, we identified the ubiquitin-specific protease USP25 as a negative regulator of IL-17-mediated signaling and inflammation. Overexpression of USP25 inhibited IL-17-triggered signaling, whereas USP25 deficiency resulted in more phosphorylation of the inhibitor IκBα and kinase Jnk and higher expression of chemokines and cytokines, as well as a prolonged half-life for chemokine CXCL1–encoding mRNA after treatment with IL-17. Consistent with that, Usp25−/− mice showed greater sensitivity to IL-17-dependent inflammation and autoimmunity in vivo. Mechanistically, stimulation with IL-17 induced the association of USP25 with the adaptors TRAF5 and TRAF6, and USP25 induced removal of Lys63-linked ubiquitination in TRAF5 and TRAF6 mediated by the adaptor Act1. Thus, our results demonstrate that USP25 is a deubiquitinating enzyme (DUB) that negatively regulates IL-17-triggered signaling.

RESULTS
USP25 suppresses IL-17-triggered signaling

K63-linked ubiquitination of TRAF6 by Act1 is reported to be critical for IL-17-triggered signaling15. The inducible kinase Ikk1 phosphorylates Act1 after stimulation with IL-17, which is important for the activation of MAPKs but not of NF-κB. Phosphorylation of Act1 mediated by Ikk1 is also required for IL-17-mediated stabilization of mRNA encoding proinflammatory cytokines16.

Protein ubiquitination is a highly regulated, dynamic process mediated by both E3 ubiquitin ligases and deubiquitinating enzymes (DUBs). The ~100 putative DUBs encoded by the human genome belong to five different subfamilies, including ubiquitin–specific proteases (USPs), ubiquitin carboxy-terminal hydrolases, ovarian tumor proteases (OUPS), metalloproteases, which have been linked to various cellular functions17. Although several DUBs have been reported to catalyze TRAF6 deubiquitination18,19, their involvement in IL-17 signaling is unknown at present. In this study, we identified USP25 as a specific, negative regulator of IL-17-mediated signaling. USP25 interacted with the adaptors TRAF5 and TRAF6 and deubiquitinated Act1-mediated K63-linked ubiquitination of TRAF5 and TRAF6, thereby turning off IL-17 signaling. Thus, USP25 is an additional regulator of IL-17-induced inflammatory responses.

The five members of the IL-17 receptor (IL-17R) family (IL-17A−IL-17E) have a SIFIR domain (‘similar expression to fibroblast growth factor receptor and IL-17R’). IL-17 signals through an IL-17RA–IL-17RC heterodimeric receptor complex11,12. After stimulation with IL-17, IL-17RA recruits the adaptor Act1 to the receptor-associated signaling complex, followed by recruitment of the ubiquitin ligase TRAF6. Act1 has a SIFIR domain and two conserved TRAF-binding sites, which are required for interaction with IL-17RA and TRAF6, respectively13,14. Act1 has been shown to function as an E3 ubiquitin ligase that catalyzes Lys63 (K63)-linked ubiquitination of TRAF6, which leads to activation of the transcription factor NF-κB and the mitogen-activated protein kinase (MAPK) Jnk15. Act1−/− mouse embryonic fibroblasts (MEFs) reconstituted with Act1(ΔU-box), a form of Act1 that lacks E3 ligase activity, fail to activate NF-κB and Jnk after stimulation with IL-17, which suggests that Act1-mediated ubiquitination of TRAF6 is critical for IL-17-triggered signaling15. The inducible kinase Ikk1 phosphorylates Act1 after stimulation with IL-17, which is important for the activation of MAPKs. Inactivation of Act1 mediated by Ikk1 is also required for IL-17-mediated stabilization of mRNA encoding proinflammatory cytokines16.

Department of Immunology and Center for Inflammation and Cancer, Texas MD Anderson Cancer Center, Houston, Texas, USA.
Correspondence should be addressed to C.D. (cdong@mdanderson.org).

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signal transduction, we did reporter assays by coexpressing various DUBs with an NF-kB–luciferase reporter construct. We found that the DUB USP25 (ref. 20) inhibited IL-17- but not TNF-induced activation of NF-kB in reporter assays in HeLa human cervical cancer cells and in 293T human embryonic kidney cells transfected to express IL-17RA and IL-17RC (Fig. 1a and Supplementary Fig. 1a). A20, another well-known DUB, inhibited signaling via both IL-17 and TNF in similar experiments (data not shown). Real-time RT-PCR analysis showed that overexpression of USP25 inhibited IL-17- but not TNF-induced expression of Cxcl1 and Il6 mRNA in HeLa cells and in 293T cells transfected to express IL-17RA and IL-17RC (Fig. 1b and Supplementary Fig. 1b). Consistent with that, but not TNF-induced phosphorylation and degradation of the NF-kB inhibitor IκBα was impaired by overexpression of USP25 (Fig. 1c). These results suggested that USP25 inhibited IL-17-induced activation of NF-kB and the expression of proinflammatory cytokines.

**USP25 deficiency enhances IL-17-mediated responses**

To investigate the physiological function of USP25 in IL-17-triggered signaling, we generated Usp25−/− mice through the use of a gene-trapping embryonic stem cell clone. We confirmed deletion of USP25 at the level of RNA and protein in various tissues and organs (Supplementary Fig. 2). Homozygous Usp25−/− mice were viable and did not show any abnormalities in growth or survival, which suggested that USP25 was dispensable for the growth and development of mice. The number and composition of cells of the immune response (including T cells, B cells, macrophages and neutrophils) in various organs (including thymus, spleen, peripheral lymph nodes, bone marrow and blood) were similar in 2- to 3-month-old Usp25−/− mice and their wild-type littermates (data not shown), which indicated that USP25 was not required for the development of various types of cells of the immune response.

We next examined by real-time RT-PCR analysis the effects of USP25 deficiency on IL-17-triggered signaling in wild-type and Usp25−/− MEFs and primary lung epithelial cells treated with IL-17 or TNF alone or IL-17 plus TNF. The expression of Cxcl1, Tnf and/or Il6 mRNA was higher in Usp25−/− cells treated with IL-17 alone or together with TNF, but not those treated with TNF alone, than in their wild-type counterparts (Fig. 2a,b). In addition, USP25 deficiency had no effect on the IL-1β-induced expression or production of IL-6 (Supplementary Fig. 3a,b). IL-17F, a member of the IL-17 family that shares 50% homology with the amino acid sequence of IL-17, signals through IL-17RA–IL-17RC and induces the expression of cytokines and chemokines alone or in synergy with TNF21.

In experiments similar to those reported above, we found that the expression of proinflammatory cytokines induced by IL-17F alone or IL-17F plus TNF was higher in Usp25−/− mice than in wild-type cells (Supplementary Fig. 3a). These results together suggested that USP25 restricted signaling via IL-17 and IL-17F in various types of cells.

**Enhanced IL-17-mediated inflammation in Usp25−/− mice**

To determine whether USP25 regulates IL-17-induced inflammatory response in vivo, we intraperitoneally injected IL-17 into wild-type and Usp25−/− mice and analyzed the expression of proinflammatory cytokines in peritoneal mesothelial cells and peritoneal cavity. The expression of Cxcl1 and Il6 mRNA was significantly higher in peritoneal cells isolated from Usp25−/− mice than those from wild-type mice (Fig. 2c). Consistent with that, the production of CXCL1 was significantly enhanced in peritoneal lavage fluid isolated from Usp25−/− mice (Fig. 2d), which indicated that USP25 physiologically regulated IL-17 signaling in vivo.

The proinflammatory cytokines and chemokines induced by IL-17 act together to amplify inflammatory response, which leads to the recruitment of neutrophils to the inflammatory sites, an idea supported by the observations that overexpression of IL-17 in lung epithelial cells or administration of IL-17 through the airways causes considerable pulmonary inflammation5,16. To investigate whether USP25 regulates IL-17-mediated airway inflammation, we treated wild-type and Usp25−/− mice with PBS or IL-17 via intranasal injection, followed by analysis of bronchoalveolar lavage fluid (BALF) and lung inflammation. There were significantly more infiltrating cells (83.5 × 104 versus 35.6 × 104) and Gr-1+CD11b+ neutrophils (76.8 × 104 versus 32.4 × 104) in BALF from Usp25−/− mice than in BALF from wild-type mice (Fig. 3a, b). Consistent with those observations, BALF from Usp25−/− mice had more CXCL1, IL-6 and TNF than did that from wild-type mice, whereas lung tissues from Usp25−/− mice had higher expression of Cxcl1, Il6 and Tnf mRNA than those from wild-type mice (Fig. 3c and data not shown). These results indicated that USP25 restricted IL-17-mediated pulmonary inflammation in vivo.
USP25 deficiency exacerbates EAE severity

IL-17 has a critical role in the development of EAE, an autoimmune disease model that resembles human multiple sclerosis. To investigate the role of USP25 in IL-17 signaling during autoimmune disease, we induced EAE in wild-type and Usp25−/− mice as described previously. We found that the severity of EAE pathology was significantly greater in Usp25−/− mice (Fig. 4a). Consistent with the greater clinical severity of disease, more macrophages and neutrophils infiltrated the central nervous system (CNS) of Usp25−/− mice, and there was significantly higher (about twofold) expression of genes encoding proinflammatory molecules, including Il6, Cxcl1 and Ccl20, in the brain and spinal cord from Usp25−/− mice (Fig. 4b,c). In contrast, the frequency and number of infiltrated CD4+IL-17+ or CD4+IFN-γ+ cells and expression of Il17a mRNA and Ifng mRNA (encoding interferon-γ (IFN-γ)) in the CNS was similar in wild-type and Usp25−/− mice (Fig. 4b,c and Supplementary Fig. 4a). In addition, the frequency and absolute number of CD4+IL-17+ cells specific for a peptide of myelin oligodendrocyte glycoprotein (amino acids 35–55) were similar in spleens from wild-type and Usp25−/− mice (Supplementary Fig. 4b). Consistent with that, splenocytes of wild-type and Usp25−/− mice produced similar amounts of IL-17 and IL-2 after stimulation with MOG (Supplementary Fig. 4c), which indicated that USP25 did not regulate IL-17 production or the T H 1 differentiation process. Although Usp25−/− splenocytes produced less IFN-γ than did wild-type splenocytes after stimulation with MOG (Supplementary Fig. 4b,c), IFN-γ expression in the CNS was similar in wild-type and Usp25−/− mice (Fig. 4b,c and Supplementary Fig. 4a). Together these data suggested that USP25 was required for the restriction of IL-17-related autoimmune diseases in the EAE disease model.

USP25 DUB activity is required for restriction of IL-17 signaling

Because IL-17-induced activation of NF-κB is responsible for expression of proinflammatory cytokines, we examined the effects of USP25 deficiency on IL-17-induced signaling. Treatment with IL-17 resulted in more phosphorylation and degradation of IκBα and phosphorylation of Jnk, but not of Act1 or the MAPKs Erk or p38, in Usp25−/− MEFs than in wild-type MEFs (Fig. 5a). In contrast, USP25 deficiency had no effect on TNF- or IL-1β-induced signaling events (Fig. 5a and Supplementary Fig. 3c).

We next determined whether the DUB activity of USP25 was required for the regulation of IL-17-induced signaling. Cys178 of USP25 is critical for the DUB activity of USP25 (ref. 24). We reconstituted Usp25−/− MEFs with wild-type USP25 or the enzyme-inactive mutant USP25(C178S) (in which the cysteine residue at position 178 is replaced with serine) by retrovirus-mediated gene transfer. Reconstitution of Usp25−/− MEFs with wild-type USP25 inhibited the induction of the expression of Cxcl1 and Il6 mRNA by IL-17 alone or by IL-17 plus TNF, but reconstitution with USP25(C178S) did not (Fig. 5b). In similar experiments, reconstitution with neither wild-type USP25 nor USP25(C178S) regulated the TNF-induced expression of Cxcl1 and Il6 (Fig. 5b). Consistent with those observations, IL-17-induced phosphorylation and degradation of IκBα and phosphorylation of Jnk, but not of Act1 or Erk, was inhibited by the reconstitution of Usp25−/− MEFs with USP25 but not by reconstitution with USP25(C178S) (Fig. 5c). To quantitatively confirm those results, we quantified phosphorylated and total IκBα and calculated the ratio of phosphorylated IκBα to total IκBα. These analyses suggested that reconstitution with USP25 almost completely inhibited IL-17-induced

Figure 3 IL-17-induced pulmonary inflammation is enhanced in the absence of USP25. (a) Infiltration of total cells or neutrophils (Gr-1+CD11b+) into the BALF of wild-type and Usp25−/− mice (n = 5 per group) given intranasal injection of PBS (50 µl) or IL-17 (1 µg in 50 µl PBS), assessed 24 h after injection. ND, not detected. (b) Histology of lung tissues from mice treated as in a, stained with hematoxylin and eosin. Original magnification, x10; scale bars, 500 µm. (c) ELISA of IL-6, Cxcl1 and TNF in BALF isolated from mice treated as in a. *P < 0.05, **P < 0.01 and ***P < 0.001 (t-test). Data are representative of two independent experiments (mean and s.d. of three replicates).
activation of NF-κB, in contrast to reconstitution with USP25(C178S) (0.89 versus 1.8 at 15 min, and 0.61 versus 8.3 at 30 min, respectively; Fig. 5c). Together these data indicated that the DUB activity of USP25 was required for the restriction of IL-17-mediated signaling.

USP25 regulates IL-17-mediated stabilization of Cxcl1 mRNA

In addition to activating the transcription of genes encoding pro-inflammatory cytokines, IL-17 acts in synergy with TNF to facilitate the stabilization of chemokine-encoding mRNA. To determine whether USP25 regulates the IL-17-mediated stabilization of chemokine-encoding mRNA, we stimulated wild-type and Usp25−/− MEFs with TNF, then treated them with actinomycin D with or without IL-17. Although the half-life of Cxcl1 and Il6 mRNA was similar in wild-type and Usp25−/− MEFs treated with actinomycin D alone, treatment with IL-17 plus actinomycin D slowed the degradation of Cxcl1 and Il6 mRNA more in Usp25−/− MEFs than in wild-type MEFs (Fig. 6a). The DUB activity of USP25 was required for this process, as indicated by the observation that the IL-17-mediated stabilization of Cxcl1 mRNA was disrupted by the reconstitution of Usp25−/− MEFs with wild-type USP25 but not by reconstitution with USP25(C178S) (Fig. 6b). To exclude the possibility that the disruption of mRNA stability by USP25 was not due to a side effect of actinomycin D treatment, we used a published HeLa Tet-Off and pTRE2-KC reporter system. In this system, in the absence of doxycycline, the tetracycline transcriptional transactivator expressed in HeLa Tet-Off cells binds to the tetracycline-responsive element upstream of the target transgene KCA4 (ref. 25) and constitutively activates its transcription. The addition of doxycycline results in inactivation of the transactivator protein, which turns off the transcription of KCA4; this allowed us to study the effects of USP25 on IL-17-mediated stabilization of KCA4 mRNA. We found that USP25 inhibited the stabilization of KCA4 mRNA by but USP25(C178S) did not (Fig. 6c). These results demonstrated that USP25 restricted IL-17-mediated stabilization of chemokine-encoding mRNA and that its DUB activity was required.

USP25 interacts with TRAF5 and TRAF6 after IL-17 treatment

Ubiquitination of TRAF proteins, particularly in the K63-linked form, has been shown to positively regulate signal transduction by various receptors that lead to the activation of NF-κB. Because USP25 negatively regulates IL-17-induced activation of NF-κB and stabilization of chemokine-encoding mRNA, which involve TRAF6 and TRAF5, respectively, we investigated whether USP25 interacted with those TRAF proteins. In transient-transfection and immunoprecipitation assays, we found that USP25 associated with TRAF3, TRAF5 and TRAF6, but not with other TRAF proteins, in 293T cells (Fig. 7a).

Figure 4 USP25 deficiency exacerbates the severity of EAE. (a) Clinical scores of Usp25−/− mice and their wild-type littermates (n = 5 per group) immunized twice with MOG peptide (amino acids 35–55), analyzed on days 1–12 after the second MOG immunization. (b) Flow cytometry of infiltrated cells of the immune response in the CNS of wild-type and Usp25−/− EAE mice at day 12 after the second immunization as in a, presented as the absolute number of cells. MΦ, macrophage; Neut, neutrophil. (c) Expression of genes in the CNS of wild-type and Usp25−/− EAE mice at day 12 after the second immunization as in a; results are presented relative to those of Actb. *P < 0.05 and **P < 0.01 (t-test). Data are representative of three independent experiments (mean and s.d.).

Figure 5 USP25 and its DUB activity are required for restriction of IL-17 signaling. (a) Immunoblot analysis of total and phosphorylated IκBα, Jnk, Erk, p38 and Act1, as well as USP25 and β-actin, in lysates of wild-type and Usp25−/− MEFs stimulated for 0–45 min (above lanes) with IL-17 (100 ng/ml) or TNF (10 ng/ml). (b) Real-time PCR analysis of Cxcl1 and Il6 mRNA in wild-type and Usp25−/− MEFs reconstituted with empty vector or vector encoding wild-type USP25 (USP25(WT)) or USP25(C178S) (USP25(CS); key), then left untreated or treated with IL-17 (50 ng/ml) or TNF (10 ng/ml) or IL-17 plus TNF; results are presented relative to those of Actb. (c) Immunoblot analysis (top) of total and phosphorylated IκBα and Act1 and phosphorylated Jnk and Erk, as well as USP25 or USP25(C178S) and β-actin, in lysates of wild-type and Usp25−/− MEFs reconstituted as in b (top) and treated for 0–10 min (above lanes) with IL-17 (50 ng/ml); below, ratio of the band intensity of phosphorylated IκBα to that of total IκBα. *P < 0.05 and **P < 0.01 (t-test). Data are representative of three independent experiments (mean and s.d. of three replicates in b).
TRAF3 has been shown to interfere with formation of the IL-17R–Act1–TRAF6 signaling complex and inhibit IL-17 signaling. We next examined interactions of the endogenous proteins in human bronchial epithelial cells (HBECs) and primary MEFs. Through the use of antibodies to various TRAF proteins for immunoprecipitation, we found that USP25 interacted with TRAF5 and TRAF6, but not with TRAF3, in HBECs and MEFs after stimulation with IL-17, whereas stimulation with TNF did not induce the USP25–TRAF5, USP25–TRAF6 or USP25–TRAF3 association (Fig. 7c and data not shown). Those results ruled TRAF3 out as a target of USP25 in IL-17-mediated signaling. Furthermore, we found that the TRAF-USP25 interaction depended on Act1, as Act1 deficiency impaired their association (Fig. 7c), which indicated that Act1 was required for USP25 to engage TRAF5 and TRAF6 in the IL-17 pathway.

USP25 contains a ubiquitin-association domain and two ubiquitin-interaction motifs, plus two peptidase domains with ubiquitin-hydrolase activity, and a coiled-coil domain. The ubiquitin-association domains and ubiquitin-interaction motifs are thought to modulate substrate recognition, whereas the peptidase domains with ubiquitin-hydrolase activity and the coiled-coil domains exert deubiquitinating activity. Through the use of deletion mutagenesis, coexpression and immunoprecipitation, we found that an intact ubiquitin-interaction motif (amino acids 91–151) of USP25 was required for its optimal interaction with TRAF6 and TRAF5 in 293T cells (Supplementary Fig. 5). In immunoprecipitation assays, we repeatedly observed that USP25 did not interact with the mutants TRAF6(C70A) or TRAF6(K124R), which cannot mediate self-ubiquitination (Supplementary Fig. 6a). To assess whether USP25 recognizes ubiquitinated TRAF6 and deubiquitinates it, we reconstituted USP25(C178S)–MEFs by transfecting them with empty vector or vector encoding Flag-tagged USP25 or USP25(C178S) and examined the IL-17-induced association between TRAF6 and USP25 or USP25(C178S) in these cells. USP25 interacted mainly with unmodified TRAF6 after IL-17 treatment, whereas the TRAF6 associated with USP25(C178S) was mostly modified (Supplementary Fig. 6b). Furthermore, treatment with IL-17 followed by N-ethylmaleimide, an inhibitor of DUBs, resulted in the association of USP25 or USP25(C178S) with modified TRAF6 and with ubiquitin-modified signals (Supplementary Fig. 6b). Thus, it is likely that treatment with IL-17 resulted in the ubiquitination of TRAF6, which recruited USP25 to remove ubiquitin chains as a feedback mechanism to restrict excessive inflammatory response.
USP25 catalyzes deubiquitination of TRAF5 and TRAF6

USP25 had no effect on the autoubiquitination of TRAF5 or TRAF6 when they were overexpressed in 293T cells (Supplementary Fig. 7a,b), which indicated that USP25 did not interfere with their E3 ligase activity. Because Act1 has been shown to induce K63-linked ubiquitination of TRAF6, which is important for IL-17-triggered activation of NF-kB and Jnk1, we investigated whether USP25 regulates this process. Act1-mediated K63-linked ubiquitination of TRAF6 was inhibited by overexpression of USP25, but not by overexpression of USP25(C178S), in 293T cells and in an in vitro deubiquitination system (Fig. 8a and Supplementary Fig. 8a). Because TRAF5 is reported to undergo post-translational modifications after IL-17 treatment, we investigated whether TRAF5 was ubiquitinated and if this modification was mediated by Act1. We found that Act1 mediated the K63-linked ubiquitination of TRAF5 (Supplementary Fig. 7c), which was substantially attenuated by USP25 but not by USP25(C178S) in 293T cells and in an in vitro deubiquitination system (Fig. 8b and Supplementary Fig. 8a). These data suggested that USP25 cleaved Act1-mediated K63-linked polyubiquitin chains from TRAF5 and TRAF6. We note that we used anti-Flag affinity gel to purify Flag-tagged USP25 in our in vitro deubiquitination experiments. Therefore, USP25 may indirectly lead to the deubiquitination of TRAF5 or TRAF6 through its tightly associated proteins.

We next examined the effect of USP25 deficiency on the ubiquitination of TRAF5 and TRAF6 after treatment with IL-17. Stimulation with IL-17 resulted in the ubiquitination of TRAF5 and TRAF6, which was enhanced in Usp25−/− MEFs (Fig. 8c). The IL-17-induced interaction between TRAF5 and the splicing factor SF2 (ASF) was potentiated in Usp25−/− MEFs, but the TRAF5–Act1 or TRAF6–Act1 association was not (Supplementary Fig. 8b). To determine whether the DUB activity of USP25 was responsible for the deubiquitination of TRAF5 and TRAF6, we reconstituted Usp25−/− MEFs by transfection of empty vector or vector encoding wild-type USP25 or USP25(C178S), and we examined the ubiquitination of TRAF5 or TRAF6 in these cells after treatment with IL-17. IL-17-induced ubiquitination of TRAF5 and TRAF6 was inhibited in Usp25−/− MEFs reconstituted with USP25 but not in those reconstituted with USP25(C178S) (Fig. 8d), which suggested that the DUB activity of USP25 was required for restriction of the IL-17-induced ubiquitination of TRAF5 and TRAF6. Together our results demonstrated that USP25 negatively regulated IL-17-triggered ubiquitination of TRAF6 and TRAF5.
DISCUSSION

It has been well documented that IL-17-triggered signaling activates NF-kB and Jnk and the expression of cytokines and chemokines, which requires ubiquitination of TRAF6. In this study, we found that overexpression of USP25 negatively regulated IL-17-triggered signaling but not TNF-triggered signaling. Conversely, USP25 deficiency resulted in hyperactivation of NF-kB and Jnk in response to stimulation with IL-17, and Usp25−/− mice showed stronger inflammatory response than those of wild-type control mice after treatment with IL-17 in vivo and were more susceptible to the induction of EAE than were their wild-type littermates. In addition to promoting TRAF6-dependent transcriptional regulation, IL-17-triggered signaling promoted the stabilization of chemokine-encoding mRNA via TRAF5, which was also restricted by USP25. These results together suggested that USP25 was required for the restriction of IL-17-induced activation of NF-kB and stabilization of chemokine-encoding mRNA, as well as inflammatory responses.

Persistent treatment with IL-17 has been reported to induce K48-linked ubiquitination and degradation of Act1 by the β-TrCP E3 ubiquitin ligase complex in a phosphorylation-dependent manner. However, it is unlikely that USP25 deubiquitates the K48-linked ubiquitination of Act1, given the following observations. First, IL-17-induced phosphorylation of Act1 would accumulate if the K48-linked ubiquitination of Act1 were blocked. In our experiments, however, we observed similar amounts of phosphorylated Act1 in IL-17-treated wild-type and Usp25−/− MEFs. Second, IL-17 treatment does not induce other forms of ubiquitination of Act1, as demonstrated by the findings that ubiquitin(K48R) (in which the lysine residue at position 48 is replaced with arginine, and thus this mutant ubiquitin cannot modify target proteins via K48) is not targeted to Act1 after IL-17 treatment and ubiquitin was not targeted to Act1 in 293T cells transfected to express both hemagglutinin-tagged ubiquitin and Flag-tagged Act1 (data not shown), which indicates that Act1 is not a substrate for USP25. Third, USP25 deficiency had no effect on the IL-17-triggered Act1-dependent activation of Erk and p38, which suggests that USP25 functions downstream of Act1.

The adaptors TRAF6 and TRAF5 function downstream of Act1 to mediate the activation of NF-kB and stability of chemokine-encoding mRNA, respectively. Act1 is an E3 ubiquitin ligase that induces K63-linked TRAF6 ubiquitination. We found that Act1 induced K63-linked ubiquitination of TRAF5. In this context, it has been observed that TRAF5 undergoes modification after IL-17 treatment. Consistent with those observations, Usp25−/− MEFs had more IL-17-induced ubiquitination of TRAF5 and TRAF6 and more potentiation of the TRAF5–ASF association than did wild-type MEFs. However, the types of ubiquitin chains on TRAF5 and TRAF6 need to be identified more fully. In addition, it is unclear how Act1-mediated ubiquitination of TRAF5 mediates IL-17-triggered signaling and how ubiquitination of TRAF5 is coupled with the recruitment of ASF. Thus, further investigation is needed to elucidate the details of the mechanisms.

TRAF6 and its ubiquitination are critical for subsequent activation of the kinase TAK1 that results in phosphorylation of Jnk and IKK for activation of the transcription factors AP-1 and NF-kB in various signaling pathways, including signaling via T cell antigen receptors, Toll-like receptors, RNA helicase RIG-I–like receptors, the IL-1 receptor and IL-17 receptors. More K63-linked ubiquitination of TRAF6 is always correlated with signaling hyperactivation. However, how ubiquitination of TRAF6 is linked to activation of TAK1 is unclear at present. It is believed that a TAK1–TAB1–TAB2 complex is recruited to the polyubiquitin chains of TRAF6 and that TAK1 is thereby activated through mutual phosphorylation. However, it has been demonstrated that TAK1 is activated by the K63-linked free ubiquitin chains synthesized by TRAF6 and a complex of the ubiquitin-conjugating enzymes Ubc13 and Mms2 (ref. 36) and/or by TRAF6-catalyzed K63-linked polyubiquitin chains that target Lys158 of TAK1 (ref. 37). However, although the mutant TRAF6(K124R), which lacks self-ubiquitination, maintains the E3 ubiquitin ligase activity that can synthesize free ubiquitin chains, it fails to mediate IL-1- or IL-17-triggered signaling (Fig. 15,22) (data not shown). One possible explanation for this is that ubiquitination of TRAF6 may activate or provide another unknown signal required for the activation of TAK1. This is not unexpected, as free ubiquitin chains synthesized by a complex of TRAF and the ubiquitin-conjugating enzyme Ubc5 and ubiquitination of the adaptor RIP by a complex of the ubiquitin ligase cIAP and Ubc5 provide two signals for TNF-induced activation of IKK36,38, and E3 ubiquitin ligase TRIM25–mediated ubiquitination of RIG-I and Ubc13–simplified free ubiquitin chains act together to facilitate the activation of RIG-I after viral infection39,40. Nonetheless, our data have demonstrated that USP25 catalyzed the deubiquitination of TRAF6, thereby ‘turning down’ IL-17-triggered signaling.

TRAF3 inhibits IL-17 signaling by competing with Act1 to interact with IL-17R41. Although USP25 interacted with TRAF3 constitutively in our overexpression system in 293T cells, treatment with IL-17 did not induce the USP25–TRAF3 association in HBEcs or MEFs. One possible explanation for this is that the recruitment of TRAF3 to IL-17R results in the dissociation of TRAF6–USP25 and TRAF5–USP25 complexes from IL-17R. However, stimulation with IL-17 induces ubiquitination of TRAF6, which provides an opportunity for the recruitment of USP25 and deubiquitination of TRAF6 by USP25. Therefore, in complement with TRAF3, the suppression of IL-17 signaling by USP25 provides a second strategy for the host to restrict IL-17-induced inflammatory response. TRAF3 has been found to be critical for both innate and adaptive immune response, in which USP25 could have a regulatory role.

In addition to IL-17 and IL-17F, IL-25 (IL-17E) also signals through Act1 and TRAF6 and is critical for allergic immune response43,44. IL-17C signaling requires Act1 and possibly TRAF6 and has been shown to regulate Th17 differentiation23. Thus, whether and how USP25 regulates signaling initiated by other members of the IL-17 family remains to be addressed. In summary, we have identified USP25 as a specific, negative regulator of IL-17 signaling. Targeting USP25 may modulate responses to IL-17 and could be beneficial in certain types of infections and cancers.

METHODS

Methods and any associated references are available in the online version of the paper.

Note: Supplementary information is available in the online version of the paper.

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AUTHOR CONTRIBUTIONS

B.Z., Xik.L. and C.D. designed the project; Xik.L. did the IL-17–induced peritoneal inflammation experiments; B.Z., X.W., Xin.L. and A.W. did the biochemical
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Hemagglutinin-tagged ubiquitin (K48 only and K63 only) were transfected those together with pGL3–NF-κB luciferase reporter (0.1 μg) and a control pRL-TK renilla luciferase reporter (0.02 μg) into HeLa cells through the use of Lipofectamine 2000. Then, 20 h after transfection, cells were stimulated with IL-17 or TNF for 8 h before luciferase assays.

Mice. Usp25 was targeted by a gene-trapping strategy (Supplementary Fig. 2). Mouse embryonic cells (clone RR805) containing trapped Usp25 alleles (BayGenomics) were microinjected into blastocysts to produce chimeras. Mice were genotyped by PCR analysis of DNA obtained from tail tissues (primers, Supplementary Table 1). Usp25+/− mice were crossed to generate age- and sex-matched Usp25+/− and Usp25−/− litters. Wild-type and Act1+/− mice have been described.23 Mice were maintained in the specific pathogen–free facility of The University of Texas MD Anderson Cancer Center. All animal experiments were in accordance with protocols approved by the Institutional Animal Care and Use Committee of The University of Texas MD Anderson Cancer Center.

IL-17–induced peritoneal or pulmonary inflammatory responses. For peritoneal inflammatory responses, age- and sex-matched Usp25+/− and Usp25−/− littersmates were injected intraperitoneally with IL-17 (0.5 μg per mouse) or PBS. Then, 24 h later, peritoneal lavage fluid was collected in 1 ml PBS. Supernatants were collected by centrifuge (1,500 r.p.m. for 5 min) and subjected to ELISA. Peritoneal mesothelial cells were isolated as described.45 The peritoneal cavity was washed with 5 ml PBS for removal of leukocytes, followed by injection of 5 ml 0.25% trypsin with massage periodically for detachment. Then, 10 min later, trypsin solutions were collected and peritoneal cavities were washed with 5 ml DMEM containing 10% FBS. Peritoneal cavities were then opened and residual mesothelial cells were collected. The expression of chemokines was measured by real-time PCR analysis.

For IL-17–induced pulmonary inflammation, age- and sex-matched Usp25+/− and Usp25−/− littersmates were treated with IL-17 (1 μg per mouse; 50 μl) or PBS (50 μl) by intranasal injection. Then, 24 h later, PBS (0.8 ml) was used to obtain BALF from mice. Supernatants were used for ELISA and precipitates were analyzed as lung-infiltrating cells. The residual lung-infiltrating cells was collected with 1 ml PBS wash. Cells were counted and then were stained with anti-Gr-1 and anti-CD11b, followed by flow cytometry. Lung tissues were collected in 1 ml ice-cold TRIzol for subsequent real-time PCR analysis or in 4% paraformaldehyde for subsequent embedment in paraffin and staining with hematoxylin and eosin.

Cell culture. Primary MEFs were prepared from embryos at embryonic days 12.5–14.5 and were cultured in DMEM containing 20% FBS, 1% streptomycin-penicillin and 10 μM β-mercaptoethanol. HeLa, HeLa Tet-Off and 293T cells were cultured in DMEM containing 10% FBS, 1% streptomycin-penicillin and 10 μM β-mercaptoethanol. HBEc3 cells were cultured in Petri dishes coated with collagen IV (5 μg/cm²; C5533; Sigma) and were maintained in DMEM containing 10% FBS, 1% streptomycin-penicillin and 10 μM β-mercaptoethanol. Primary lung epithelial cells and leukocytes were isolated as described.44,46

Disperse solution (2 ml at 3.6 unit/ml; 17105–01; Gibco) was instilled into the lungs through a tracheal catheter. Lungs were removed from mice and incubated in dispase solution for 1 h at room temperature. Lung tissues were microdissected in dispase solution and cell suspensions were filtered through nylon monofilament, then recovered cells were resuspended in DMEM containing 10% FBS. For isolation of primary lung leukocytes, cells were incubated with anti–CD45 microbeads and CD45+ cells were selected by AutoMACS (Miltenyi Biotec). For preparations of primary lung epithelial cells, cells were incubated for 30 min at 4 °C with rat anti–CD32–CD16 (553142; BD Biosciences) and anti–CD45 (553076; BD Biosciences), followed by incubation with anti-rat IgG microbeads and negative selection by AutoMACS. Cells were resuspended in DMEM containing 10% FBS, 1% streptomycin-penicillin and 10 μM β-mercaptoethanol and were seeded into 48-well plates at a density of 1 × 10⁴ cells per well for overnight culture, followed by various treatments.

MOG immunization and induction of EAE. MOG immunization and EAE induction were done as described.22–23. Wild-type and Usp25−/− littersmates 3 months of age were immunized twice with 300 μg MOG peptide (amino acids 35–55; MEVGWFRSPFSRQVHLRNGK) emulsified in complete Freund’s adjuvant, followed by intraperitoneal injection of pertussis toxin (100 μl) 1 d after immunization. Disease scores were assigned on a scale of 0–5 as follows: 0, none; 0.5, limp tail or waddling gait with tail tonicity; 2, wobbly gait; 3, hindlimb paralysis; 4, hindlimb and forelimb paralysis; 5, death.

CNS mRNA analysis. Brain and spinal cord were obtained from mice with EAE on day 12 after the second immunization and single cell suspension was prepared. An aliquot of these cells was obtained and mRNA was isolated with TRizol reagent (Invitrogen), then cDNA was synthesized and real-time PCR was done.

Antibodies and reagents. Mouse control IgG (sc-2025; Santa Cruz Biotechnology) and rabbit control IgG (12-370; Millipore), horseradish peroxidase–conjugated goat-anti mouse or rabbit IgG (PAI-8671 and SA1-9510; Thermo Scientific), horseradish peroxidase–conjugated anti-Myc (sc-40; Santa Cruz Biotechnology), mouse anti-Flag (F1804; Sigma), anti-ubiquitin (sc-8017; Santa Cruz Biotechnology), anti-hemagglutinin (MMS-101R; Covance), anti-IkBα (sc-371; Santa Cruz Biotechnology), anti–TRAF5 (sc-7450; Santa Cruz Biotechnology), rabbit antibody to K63-linked ubiquitin (05-1308; Millipore), anti-Erk (sc-94; Santa Cruz Biotechnology), anti-Jnk (sc-474; Santa Cruz Biotechnology), anti-p38 (sc-7149; Santa Cruz Biotechnology), antibody to Erk phosphorylated at Thr202 and Tyr204 (4370S; Cell Signaling Technology), antibody to Jnk phosphorylated at Thr183 and Tyr185 (9251S; Cell Signaling Technology), antibody to p38 phosphorylated at Thr180 and Tyr182 (4631L; Santa Cruz Biotechnology), gene–TRAF6 (sc-7221; Santa Cruz Biotechnology), anti–TRAF5 (sc-7220; Santa Cruz Biotechnology), anti–Act1 (sc-1144A; Santa Cruz Biotechnology) and anti–β-actin (A2066; Sigma) were used. Mouse anti–TRAF6 was a gift from H.-B. Shu. Rabbit anti-USP25 was described and was provided by G. Marfany. IL-1 and TNF were from Peprotech, and IL-17 and IL-17F were from BD Biosciences.

 Constructs. Mammalian expression plasmids for Flag- or Myc-tagged USP25 and its truncated mutants were constructed in the pcDNA6.0 vector. Plasmids encoding TRAF1–TRAF6 were provided by H.-B. Shu. Flag-tagged USP25(C178S) was used with a site-directed mutagenesis kit (200519; Stratagene). The pcDNA encoding IL-17RA and IL-17RC has been described.21,22. Hemagglutinin-tagged ubiquitin (K48 only and K63 only) were provided by Z. Chen.

 Quantitative real-time PCR and ELISA. Cells treated with various stimuli were collect in TRIzol (15596-018; Invitrogen) and first-strand cDNA was synthesized with a reverse-transcription kit (4368814; Invitrogen). For analysis of KC4 mRNA in Tet-Off cells, prepared mRNA was treated for 30 min with RNase-free DNase, followed by heating for 10 min at 65 °C, and oligo(dT) and reverse-transcription primers for KC4 RT (Supplementary Table 1) were used for first-strand cDNA synthesis. Gene expression was assessed with a Bio-Rad iCycler Optical system with an iQ SYBR Green Real-Time PCR kit (Bio-Rad Laboratories). Data were normalized to the expression of the gene encoding β-actin. Gene-specific primers are in Supplementary Table 1. TNF, IL-6 and CXCL1 in samples were measured by standard ELISA (BD Biosciences).

Coimmunoprecipitation, immunoblot and ubiquitination assays. Coimmunoprecipitation and immunoblot and ubiquitination assays were done as described.44,46. Non-denaturing immunoprecipitation was done in lysis buffer (20 mM Tris, pH 7.4, 150 mM NaCl, 1 mM EDTA, 20 mM N-ethylmaleimide) and 1% Nonidet-P40, and immunoprecipitates were re-extracted in lysis buffer containing 1% SDS and denatured by heating for 5 min. Supernatants were diluted with lysis buffer until the concentration of SDS was decreased to 0.1%, followed by reimmunoprecipitation with the appropriate antibodies. Immunoprecipitates were analyzed by immunoblot with anti-ubiquitin or antibody specific for K63-linked ubiquitin. For in vitro
ubiquitination experiments, proteins were expressed with a TNT Quick Coupled Transcription/Translation Systems kit (Promega). Ubiquitination was analyzed with a ubiquitination kit according to the protocols recommended by the manufacturer (Enzo Life Sciences). Flag-tagged wild-type USP25 and USP25(C178S) were purified by immunoprecipitation with anti-Flag gel (A2220; Sigma) from lysates of 293T cells transfected to express Flag-tagged wild-type USP25 and USP25(C178S), followed by elution with Flag peptide (50 µg/ml; F4799; Sigma). In vitro deubiquitination assays were done as described46. The in vitro ubiquitination reactions were terminated by the addition of EDTA (10 mM). Terminated reaction mixtures were incubated with Flag-tagged USP25 or USP25(C178S) for 2 h at 37 °C and overnight at 16 °C before immunoblot analysis.

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