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Negative regulation of IL-17-mediated signaling and inflammation by ubiquitin-specific protease 25

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Abstract

Interleukin 17 (IL-17) plays an important role in infection and autoimmunity; how it signals remains poorly understood. In this study, we identified ubiquitin-specific protease 25 (USP25) as a negative regulator of IL-17-mediated signaling and inflammation. Overexpression of USP25 inhibited IL-17-triggered signaling, while USP25 deficiency resulted in increased phosphorylation of IκBα and Jnk, increased expression of chemokines and cytokines as well as prolonged half-life of *Cxcl1* mRNA following IL-17 treatment. Consistently, *Usp25*$^{-/-}$ mice exhibited increased sensitivity to IL-17-dependent inflammation and autoimmunity *in vivo*. Mechanistically, IL-17 stimulation induced the association of USP25 with TRAF5 and TRAF6 and USP25 induced removal of Act1-mediated K63-linked ubiquitination in TRAF5 and TRAF6. Thus, our results demonstrate that USP25 is a deubiquitinating enzyme (DUB) that negatively regulates IL-17-triggered signaling.

Interleukin 17 (IL-17, also called IL-17A) is a pro-inflammatory cytokine that plays critical roles in host defense to infection, autoimmunity and tumorigenesis[1]. *Il17a*$^{-/-}$ mice are resistant to experimental autoimmune encephalomyelitis (EAE), but susceptible to *Candida albicans* infection[2-4]. IL-17 is the signature cytokine produced by the T helper 17 (T_H17) cell subset[5-7]. A number of innate and adaptive immune cell subsets are also capable of producing IL-17, including $CD8^+$ T cells, γδ T cells, monocytes, natural killer (NK) cells and lymphoid tissue inducer-like cells[8]. Upon IL-17 stimulation, tissue resident cells produce various pro-inflammatory cytokines and chemokines such as CXCL1, IL-6, G-CSF and CCL20, which collaborate to induce the inflammatory response and chemotaxis of neutrophils and other myeloid cells to the inflamed sites[9]. In addition, IL-17-triggered signaling prolongs the half-life of pro-inflammatory cytokine mRNA and acts

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AUTHOR CONTRIBUTIONS

B.Z., X.L. and C.D. designed the project. X.L. did the IL-17-induced peritoneal inflammation experiments. B.Z. X.W. X.L, A.W. did the biochemical experiments and EAE analysis. B.Z. and S.C. prepared MEFs and performed the IL-17 induced lung inflammation experiments. B.Z. and J.R. prepared lung epithelial cells. B.Z. and C.D. wrote and revised the manuscript.

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synergistically with other inflammatory stimuli, such as TNF and TLR ligands, to induce more extensive production of pro-inflammatory cytokines[10].

The five members of the IL-17 receptor (IL-17R) family (from IL-17RA to IL-17RE) express the SEFIR (FGF receptor and IL-17R) domain. IL-17 signals through an IL-17RA/IL-17RC heterodimeric receptor complex[11,12]. Upon IL-17 stimulation, IL-17RA recruits the Act1 adaptor to the receptor-associated signaling complex, followed by recruitment of TRAF6. Act1 has a SEFIR domain and two conserved TRAF-binding sites, which are required for interaction with IL-17RA and TRAF6, respectively[13,14]. Act1 was recently shown to function as an E3 ubiquitin ligase that catalyzes K63-linked ubiquitination of TRAF6, leading to the activation of NF-κB and Jnk[15]. *Act1*$^{-/-}$ MEFs reconstituted with Act1(ΔU-box), which lacks its E3 ligase activity, failed to activate NF-κB and Jnk after IL-17 stimulation, suggesting that Act1-mediated ubiquitination of TRAF6 is critical for IL-17-triggered signaling[15]. IKKi phosphorylates Act1 upon IL-17 stimulation, which is important for MAPKs, but not NF-κB activation. IKKi-mediated phosphorylation of Act1 is also required for IL-17-mediated mRNA stabilization of pro-inflammatory cytokines[16].

Protein ubiquitination is a highly regulated, dynamic process mediated by both E3 ubiquitin ligases and deubiquitinating enzymes (DUBs). About 100 putative DUBs in the human genome belong to five different subfamilies, including ubiquitin-specific proteases (USPs), ubiquitin carboxy-terminal hydrolases, ovarian tumor (OTU) domain-containing proteases, the Machado-Joseph disease proteases and the metallo-proteases, which have been implicated in various cellular functions[17]. Although several DUBs have been reported to catalyze deubiquitination of TRAF6[18,19], their involvement in IL-17 signaling is currently unknown.

In this study, we identified USP25 as a specific, negative regulator for IL-17-triggered signaling. USP25 interacts with TRAF5 and TRAF6 and deubiquitinates Act1-mediated K63-linked ubiquitination of TRAF5 and TRAF6, thereby turning off IL-17 signaling. Thus, USP25 is a new regulator of IL-17-induced inflammatory response.

RESULTS

USP25 is a suppressor of IL-17-triggered signaling

K63-linked ubiquitination of TRAF6 by Act1 was reported to be critical for IL-17-mediated activation of NF-κB and expression of pro-inflammaroty cytokines[15]. To identify new regulators of IL-17 signal transduction, we performed reporter assays by co-expression of various deubiquitinating enzymes (DUBs) with an NF-κB-luciferase reporter construct. We found that the DUB, USP25[20] inhibited IL-17-but not TNF-induced activation of NF-κB in reporter assays in HeLa cells and in 293T cells transfected with IL-17RA and IL-17RC (293T-IL-17RA/C) (**Fig. 1a** and **Supplementary Fig. 1a**). A20, another well-known DUB, inhibited both IL-17 and TNF signaling 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-IL-17RA/C cells (**Fig. 1b** and **Supplementary Fig. 1b**). Consistently, IL-17- but not TNF-induced phosphorylation and degradation of IκBα was impaired by overexpression of USP25 (**Fig.**

1c). These results suggest that USP25 inhibits IL-17-induced activation of NF-κB and the expression of pro-inflammatory cytokines.

USP25 deficiency enhances IL-17-mediated responses

To investigate the physiological function of USP25 in IL-17-triggered signaling, we generated *Usp25*$^{-/-}$ mice by using a gene-trapping ES clone (see Methods). Deletion of USP25 was confirmed at the RNA and protein levels in various tissues and organs (**Supplementary Fig. 2**). Homozygous *Usp25*$^{-/-}$ mice were viable and did not show any abnormalities in growth and survival, suggesting that USP25 is dispensable for growth and development in mice. Immune cell number and composition including T cells, B cells, macrophages and neutrophils in various organs including thymus, spleen, peripheral lymph nodes, bone marrow and blood were comparable between 2-3-month-old *Usp25*$^{-/-}$ mice and the wild-type littermates (data not shown), indicating that USP25 is not required for development of various types of immune cells.

We next examined the effects of USP25 deficiency on IL-17-triggered signaling in wild-type and *Usp25*$^{-/-}$ mouse embryonic fibroblasts (MEFs) and primary lung epithelial cells treated with IL-17 or TNF alone or IL-17 plus TNF by real-time RT-PCR analysis. Compared to wild-type cells, the expression of *Cxcl1*, *Tnf* and/or *Il6* mRNA was enhanced in *Usp25*$^{-/-}$ cells treated with IL-17 alone or in synergy with TNF but not with TNF alone (**Fig. 2a,b**). In addition, USP25 deficiency had no effect on IL-1β-induced expression and production of IL-6 (**Supplementary Fig. 3a,b**). IL-17F, an IL-17 family member sharing 50% of the amino acid sequence homology with IL-17, signals through IL-17RA/RC and induces expression of cytokines and chemokines alone or in synergy with TNF[21]. In similar experiments, we found that IL-17F- or IL-17F plus TNF-induced expression of pro-inflammatory cytokines was increased in *Usp25*$^{-/-}$ cells compared to wild-type cells (**Fig. 2a,b**). These data together suggest that USP25 restricts IL-17 and IL-17F signaling in various types of cells.

Enhanced IL-17-mediated inflammatory responses 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 pro-inflammatory cytokines in peritoneal mesothelial cells and peritoneal cavity. The expression of *Cxcl1* and *Il6* mRNA was significantly increased in peritoneal cells isolated from *Usp25*$^{-/-}$ mice compared to those from wild-type mice (**Fig. 2c**). Consistently, the production of CXCL1 was significantly enhanced in the peritoneal lavage fluid isolated from *Usp25*$^{-/-}$ mice (**Fig. 2d**), indicating that USP25 physiologically regulates IL-17 signaling *in vivo*.

The pro-inflammatory cytokines and chemokines induced by IL-17 collaborate to amplify inflammatory response, leading to 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 airway causes strong pulmonary inflammation[5,16]. To investigate whether USP25 regulates IL-17-mediated airway inflammation, wild-type and *Usp25*$^{-/-}$ mice were treated 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 (83.5×10^4 v.s. 35.6×10^4) and $Gr1^+CD11b^+$ neutrophils (76.8×10^4 v.s. 32.4×10^4) in the BALF from *Usp25*$^{-/-}$ mice than wild-type mice (**Fig. 3a**). Results from histological analysis of lung tissue showed increased inflammation in the lung of *Usp25*$^{-/-}$ mice (**Fig. 3b**). Consistent with these observations, BALF from *Usp25*$^{-/-}$ mice contained higher levels of CXCL1, IL-6 and TNF than that from wild-type mice, while lung tissues from *Usp25*$^{-/-}$ mice showed elevated expression of *Cxcl1*, *Il6* and *Tnf* mRNA compared to wild-type mice (**Fig. 3c** and data not shown). These results indicate that USP25 restricts IL17-mediated pulmonary inflammation *in vivo*.

USP25 deficiency aggravates EAE severity

IL-17 plays a critical role in the development of experimental autoimmune encephalomyelitis (EAE), an autoimmune disease model that resembles human multiple sclerosis[2]. To examine the role of USP25 in IL-17 signaling during autoimmune disease, we induced EAE in wild-type and USP25-deficient mice as previously described[22,23]. The severity of EAE pathology was significantly aggravated in *Usp25*$^{-/-}$ mice (**Fig. 4a**). Consistent with the clinical severity of disease, there were increased numbers of macrophages and neutrophils infiltrating into the central nervous system (CNS) of *Usp25*$^{-/-}$ mice and significantly higher (~2 folds) expression of proinflammatory genes, including *Il6*, *Cxcl1* and *Ccl20* in the brain and spinal cord from *Usp25*$^{-/-}$ mice (**Fig. 4b,c**). In contrast, the percentages or cell numbers of infiltrated $CD4^+IL\text{-}17^+$ or $CD4^+IFN\text{-}\gamma^+$ cells or expression levels of *Il17a* and *Ifng* mRNA in the CNS were comparable between the wild-type and *Usp25*$^{-/-}$ mice (**Fig. 4b,c** and **Supplementary Fig. 4a**). In addition, the percentage and absolute numbers of MOG-specific $CD4^+IL\text{-}17^+$ cells were comparable in the spleens from wild-type and *Usp25*$^{-/-}$ mice (**Supplementary Fig. 4b**). Consistently, splenocytes of wild-type and *Usp25*$^{-/-}$ mice produced similar levels of IL-17 and IL-2 upon MOG stimulation (**Supplementary Fig. 4c**), indicating that USP25 did not regulate IL-17 production or T_H17 differentiation process. Although *Usp25*$^{-/-}$ splenocytes produced less IFN-γ than wild-type splenocytes upon MOG stimulation (**Supplementary Fig. 4b,c**), IFN-γ expression in CNS was comparable between wild-type and *Usp25*$^{-/-}$ mice (**Fig. 4b,c** and **Supplementary Fig. 4a**). Taken together, these data suggest that USP25 is required for 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 pro-inflammatory cytokines, we examined the effects of USP25 deficiency on IL-17-induced signaling. IL-17 treatment resulted in enhanced phosphorylation and degradation of IκBα and phosphorylation of Jnk, but not Act1, Erk or p38 in USP25-deficient MEFs compared to 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 examined whether the DUB activity of USP25 is required for regulating IL-17-induced signaling. The Cys178 residue of USP25 is critical for the DUB activity of USP25[24]. *Usp25*$^{-/-}$ MEFs were reconstituted with wild-type USP25 or an enzyme inactive mutant, USP25(C178S), by retrovirus-mediated gene transfer. Reconstitution of USP25, but

not USP25(C178S) in *Usp25*$^{-/-}$ MEFs inhibited IL-17- or IL-17 plus TNF-induced expression of *Cxcl1* and *Il6* mRNA (**Fig. 5b**). In similar experiments, reconstitution of neither wild-type USP25 nor USP25(C178S) regulated TNF-induced expression of *Cxcl1* and *Il6* (**Fig. 5b**). Consistent with these observations, IL-17-induced phosphorylation and degradation of IκBα and phosphorylation of Jnk but not Act1 or Erk was inhibited by reconstitution of USP25 but not USP25(C178S) in *Usp25*$^{-/-}$ MEFs (**Fig. 5c**). To quantitatively confirm these results, we quantified the p-IκBα and IκBα bands and calculated the ratios of p-IκBα/IκBα. The analysis suggested that reconstitution of USP25 almost completely inhibited IL-17-induced activation of NF-κB compared to USP25(C178S) (0.89 v.s 1.8 at 15 min, 0.61 v.s. 8.3 at 30 min, respectively) (**Fig. 5c**).Taken together, these data indicate that the DUB activity of USP25 is required for restriction of IL-17-mediated signaling.

USP25 regulates IL-17-mediated chemokine mRNA stability

In addition to activating the transcription of pro-inflammatory cytokine genes, IL-17 synergizes with TNF to facilitate the stabilization of chemokine mRNA. To examine whether USP25 regulates IL-17-mediated stabilization of chemokine mRNA, wild-type and USP25-deficient MEFs were stimulated with TNF, followed by actinomycin D with or without IL-17 treatment. Although the half-life of *Cxcl1* and *Il6* mRNA was similar between wild-type and USP25-deficient MEFs treated with actinomycin D alone, IL-17 plus actinomycin D treatment slowed down the degradation of *Cxcl1* and *Il6* mRNA more significantly in USP25-deficient MEFs compared to wild-type MEFs (**Fig. 6a**). The DUB activity of USP25 was required for this process, as indicated by the observation that IL-17-mediated stabilization of *Cxcl1* mRNA was disrupted by reconstitution of wild-type but not USP25(C178S) in *Usp25*$^{-/-}$MEFs (**Fig. 6b**). To exclude the possibility that the disruption of mRNA stability by USP25 was not due to the side effect of actinomycin D treatment, we adopted the HeLa Tet-Off and pTRE2-KCΔ4 reporter system as previously reported[25]. In the absence of doxycycline, the tetracycline transcriptional transactivator expressed in HeLa Tet-Off cells binds to the tetracycline-responsive element upstream of KCΔ4 and constitutively activates its transcription. Addition of doxycycline results in inactivation of the transactivator protein and turns off the transcription of KCΔ4, which allowed us to study the effects of USP25 on IL-17-mediated stabilization of KCΔ4 mRNA. USP25 but not USP25(C178S) inhibited the stabilization of KCΔ4 mRNA (**Fig. 6c**). These results demonstrate that USP25 restricts IL-17-mediated stabilization of chemokine mRNA and its DUB activity is required.

USP25 interacts with TRAF5 and TRAF6 after IL-17 stimulation

Ubiquitination of TRAF proteins, particularly the K63-linked form, has been shown to positively regulate signal transduction by multiple receptors leading to NF-κB activation. Because USP25 negatively regulates IL-17-induced activation of NF-κB and stabilization of chemokine mRNA, which involves TRAF6 and TRAF5, respectively[26], we examined whether USP25 interacts with these TRAF proteins. In our transient transfection and immunoprecipitation assays, we found that USP25 associated with with TRAF3, TRAF5 and TRAF6, but not with other TRAF proteins in 293T cells (**Fig. 7a**). TRAF3 was

demonstrated to interfere with the formation of IL-17R-Act1-TRAF6 signaling complex and to inhibit IL-17 signaling[27]. We next examined interactions of the endogenous proteins in human bronchial epithelial cells (HBECs) or primary MEFs. Using antibodies to various TRAF for immunoprecipitation revealed that USP25 interacted with TRAF5 and TRAF6, but not with TRAF3 in HBECs or MEFs after IL-17 stimulation, while TNF stimulation induced neither USP25-TRAF5, USP25-TRAF6 nor USP25-TRAF3 association (**Fig. 7b,c** and data not shown). These results ruled out TRAF3 as atarget of USP25 in IL-17-mediated signaling. Furthermore, we found that TRAF-USP25 interaction depended on Act1, as Act1 deficiency impaired their association (**Fig. 7c**), indicating that Act1 was required for USP25 to engage TRAF5 and TRAF6 in the IL-17 pathway.

USP25 contains a ubiquitin-acsociatign domain (UBA) and two ubiquitin-interaction motifs (UIM), two peptidase domains (UCH) containing ubiquitin hydrolase activity, and a coiled-coil domain. The UBA-UIM domains are thought to modulate substrate recognition, while the UCH-coil domains exert deubiquitinating activity. Using deletion mutagenesis and co-expression and immunoprecipitaion experiments, we found that the intact UIM (aa91-151) domain of USP25 was required for optimal association with TRAF6 and TRAF5 in 293T cells (**Supplementary Fig. 5**). Using immunoprecipitation assays we consistently observed that USP25 did not interact with TRAF6(C70A) or TRAF6(K124R) mutants, which cannot mediate self ubiquitination[27] (**Supplementary Fig. 6a**). To test if USP25 recognizes ubiquitinated TRAF6 and deubiquitinates it, we reconstituted *Usp25*$^{-/-}$ MEFs with empty vector, Flag-tagged USP25 or USP25(C178S) and examined 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 USP25(C178S) associated TRAF6 was mostly modified (**Supplementary Fig. 6b**). Furthermore, treatment with IL-17 and N-Ethylmaleimide (NEM), an inhibitor of DUBs, resulted in association of USP25 or USP25(C178S) with modified TRAF6 and with ubiquitin-modified signals (**Supplementary Fig. 6b**). Thus, it is likely that IL-17 treatment results in ubiquitination of TRAF6, which recruits USP25 to remove ubiquitin chains as a feedback mechanism to restrict excessive inflammatory response.

USP25 deubiquitinates Act1-mediated ubiquitination of TRAF5 and TRAF6

USP25 had no effect on auto-ubiquitination of TRAF5 or TRAF6 when they were overexpressed in 293 T cells (**Supplementary Fig. 7a,b**), indicating 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-κB and Jnk[15], we investigated whether USP25 regulates this process. Act1-mediated K63-linked ubiquitination of TRAF6 was inhibited by overexpression of USP25, but not USP25(C178S) in 293T cells or in an *in vitro* deubiquitination system (**Fig. 8a** and **Supplementary Fig. 8a**). Because TRAF5 was reported to undergo posttranslational modifications after IL-17 treatment[25], we tested if TRAF5 was ubiquitinated, and if this modification was mediated by Act1. We found that Act1 mediated K63-linked ubiquitination of TRAF5 (**Supplementary Fig. 7c**), which was substantially attenuated by USP25 but not USP25(C178S) in 293T cells or in an *in vitro* deubiquitination system (**Fig. 8b** and **Supplementary Fig. 8a**). These data suggest that USP25 cleaves Act1-mediated K63-linked polyubiquitin chains from TRAF5

and TRAF6. It should be noted that anti-Flag agarose was used to purify Flag-tagged USP25 in our *in vitro* deubiquitination experiments. Therefore, USP25 may indirectly lead to deubiquitination of TRAF5 or TRAF6 through its tightly associated proteins.

We next examined the effect of USP25 deficiency on ubiquitination of TRAF5 and TRAF6 after IL-17 treatment. IL-17 stimulation results in 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 but not TRAF5-Act1 or TRAF6-Act1 association was potentiated in *Usp25*$^{-/-}$ MEFs (**Supplementary Fig. 8b**). To determine whether the DUB activity of USP25 was responsible for the deubiquitination of TRAF5 and TRAF6, we reconstituted empty vector, USP25 or USP25(C178S) into *Usp25*$^{-/-}$ MEFs and examined the ubiquitination of TRAF5 or TRAF6 in these cells after IL-17 treatment. IL-17-induced ubiquitination of TRAF5 and TRAF6 was inhibited in *Usp25*$^{-/-}$ MEFs reconstituted with USP25 but not USP25(C178S) (**Fig. 8d**), suggesting that USP25 DUB activity is required for restriction of IL-17-induced ubiquitination of TRAF5 and TRAF6. Taken together, our results demonstrate that USP25 negatively regulates IL-17-triggered ubiquitination of TRAF6 and TRAF5.

DISCUSSION

It has been well documented that IL-17-triggered signaling activates NF-κB, Jnk and expression of cytokines and chemokines, which requires ubiquitination of TRAF6. In this study, we found that overexpression of USP25 negatively regulated IL-17- but not TNF-triggered signaling. Conversely, USP25 deficiency resulted in hyper-activation of NF-κB and Jnk in response to IL-17 stimulation and *Usp25*$^{-/-}$ mice exhibited stronger inflammatory response than wild-type controls after IL-17 treatment *in vivo* and were more susceptible to EAE induction than the wild-type littermates. In addition to TRAF6-dependent transcriptional regulation, IL-17-triggered signaling promotes stabilization of chemokine mRNA via TRAF5, which was also restricted by USP25. These results together suggest that USP25 is required for restriction of IL-17-induced activation of NF-κB, stabilization of chemokine mRNA as well as inflammatory responses.

Persistent IL-17 treatment was reported to induce K48-linked ubiquitination and degradation of Act1 by β-TrCP complex in a phosphorylation-dependent manner[28]. However, it is unlikely that USP25 deubiquitinates K48-linked ubiquitination of Act1. First, IL-17-induced phosphorylation of Act1 would accumulate if the K48-linked ubiquitination of Act1 is blocked. In our experiments, however, we observed comparable 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 results that ubiquitin(K48R) is not targeted to Act1 after IL-17 treatment[28] and ubiquitin was not targeted to Act1 in co-transfected 293T cells (data not shown), indicating that Act1 is not a substrate for USP25. Third, USP25 deficiency had no effect on IL-17-triggered Act1-dependent activation of Erk and p38, suggesting that USP25 functions downstream of Act1.

TRAF6 and TRAF5 are two adaptor proteins that function downstream of Act1 to mediate activation of NF-κB and chemokine mRNA stability, respectively[26]. Act1 is an E3 ubiquitin

ligase that induces K63-linked ubiquitination of TRAF6[15]. 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[25]. Consistent with these observations, IL-17-induced ubiquitination of TRAF5 and TRAF6 was increased and TRAF5-ASF association was potentiated in *Usp25*$^{-/-}$ MEFs compared to wild-type MEFs. However, the types of ubiquitin chains on TRAF5 and TRAF6 need to be further determined. In addition, it is unclear how Act1-mediated ubiquitination of TRAF5 mediates IL-17-triggered signaling and how TRAF5 ubiquitination is coupled with recruitment of ASF. Thus, further investigations are required to elucidate the detailed mechanisms.

TRAF6 and its ubiquitination are critical for subsequent activation of TAK1 that results in phosphorylation of Jnk and IKK for activation of the transcription factors AP-1 and NF-κB in various signaling pathways including TCR, TLR, RLR, IL-1R and IL-17R signaling[29]. Increased K63-linked ubiquitination of TRAF6 is always correlated with hyperactivation of signaling[19,30-32]. However, how TRAF6 ubiquitination is linked to TAK1 activation is unclear at this moment. It was believed that a TAK1-TAB1-TAB2 complex was recruited to polyubiquitin chains of TRAF6 and TAK1 was thereby activated through mutual phosphorylation[33-35]. Recently however, it has been demonstrated that TAK1 is activated by the K63-linked free ubiquitin chains synthesized by TRAF6 and UbcH13-Mms2[36] and/or by TRAF6-catalyzed K63-linked polyubiquitin chains targeted at Lys158 of TAK1[37]. However, although TRAF6(K124R), which loses self-ubiquitination, maintains the E3 ubiquitin ligase activity that can synthesize free ubiquitin chains[27], it failed to mediate IL-1 or IL-17-triggered signaling (our unpublished observations)[15,27]. One possible explanation for this is that ubiquitination of TRAF6 may activate or provide another currently unknown signal that is required for TAK1 activation. This is not surprising, as free ubiquitin chains synthesized by TRAF-Ubc5 and ubiquitination of RIP by cIAP-Ubc5 provide two signals for TNF-induced IKK activation[36,38] and TRIM25-mediated ubiquitination of RIG-I and Ubc13-synthesized free ubiquitin chains collaborate to facilitate activation of RIG-I after viral infection[39,40]. Nonetheless, our data demonstrate that USP25 catalyzes deubiquitination of TRAF6, thereby turning down IL-17-triggered signaling.

TRAF3 inhibits IL-17 signaling via competing with Act1 to interact with IL-17R[41]. Although USP25 interacted with TRAF3 constitutively in overexpression system in 293T cells, IL-17 treatment did not induce USP25-TRAF3 association in HBECs or MEFs. One possible explanation for this is that recruitment of TRAF3 to IL-17R results in disassociation of TRAF6-USP25 and TRAF5-USP25 complexes from IL-17R. On the other hand, IL-17 stimulation induces ubiquitination of TRAF6, which provides an opportunity for USP25 recruitment and deubiquitination of TRAF6 by USP25. Therefore, in complement with TRAF3, the suppression of IL-17 signaling by USP25 provides a second strategy for host to restrict IL-17-induced inflammatory response. TRAF3 has been found to be critical for both innate and adaptive immune response[42], in which USP25 could play a regulatory role.

In addition to IL-17 and IL-17F, IL-25 (also called IL-17E) also signals through Act1 and TRAF6 and is critical for allergic immune response[43,44]. IL-17C signaling requires Act1 and possibly TRAF6 and has been shown to regulate T_H17 differentiation[23]. Thus, whether and

how USP25 regulates signaling initiated by other IL-17 family members 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.

METHODS

Reporter assay

To identify DUBs that modulate IL-17 signaling, we constructed a pool of expression plasmids encoding DUBs and co-transfected them with pGL3-NF-κB luciferase (0.1 μg) reporter and a control pRL-TK Renilla luciferase reporter (0.02 μg) into HeLa cells by Lipofectamine 2000. Twenty hours after transfection, cells were stimulated with IL-17 or TNF for 8 hours before luciferase assays were performed.

Mice

Usp25 gene was targeted by a gene trapping strategy (**Supplementary Figure 2**). Mouse embryonic cells (clone RRS805) containing trapped *Usp25* alleles were obtained from BayGenomics and were microinjected into blastocysts to produce chimeras. Mice were genotyped by PCR analysis of DNA obtained from tail tissues (for primers, see **Supplementary Table**). The $Usp25^{+/-}$ mice were crossed to get age and sex matched $Usp25^{+/+}$ or $Usp25^{-/-}$ littermates. Wild-type and $Act1^{-/-}$ mice were previously 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 $Usp25^{-/-}$littermates were injected intraperitoneally with IL-17 (0.5 μg per mouse) or PBS. Twenty-four hours later, peritoneal lavage fluids were collected in 1 ml PBS. The supernatant was collected by centrifuge (1500 rpm, 5min) and subjected to ELISA analysis. The peritoneal mesothelial cells were isolated as previously described[45]. Briefly, the peritoneal cavity was washed by 5 ml PBS to remove leukocytes followed by injection of 5 ml 0.25% trypsin with massage periodically for detachment. Ten minutes later, the trypsin solutions were collected and peritoneal cavities were washed with 5 ml DMEM containing 10% FBS. The 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 $Usp25^{-/-}$ littermates were treated with IL-17 (1 μg per mouse, 50 μl) or PBS (50 μl) via intranasal

injection. Twenty-four hours later, PBS (0.8 ml) was used to obtain BALF though the trachea. The supernatants were saved for ELISA analysis and the precipitants were saved as lung infiltrating cells. The residual lung infiltrating cells was collected with 1 ml PBS wash. The cells were counted and stained with anti-Gr1 and anti-CD11b followed by flow cytometry analysis. 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 E12.5-14.5 embryos and 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. HBEC were cultured in collagen IV (5 μg/cm^2, Sigma, C5533)-coated Petri dish and maintained in DMEM containing 10% FBS, 1% streptomycin-penicillin and 10 μM β-mercaptoethanol. Primary lung epithelial cells and leukocytes were isolated as previously described[44,46]. In brief, two milliliters of dispase solution (3.6 unit/ml, GIBCO, 17105-41) was instilled into the lungs through a tracheal catheter. The lungs were removed from mice and incubated in dispase solution at room temperature for 1 hour. The lung tissues were microdissected in the dispase solution and the cell suspension was filtered through nylon monofilament and the recovered cells were resuspended in DMEM containing 10% FBS. For primary lung leukocytes isolation, the cells were incubated with anti-CD45 microbeads and the CD45$^+$ cells were selected by Automacs (Miltenyi Biotec). For primary lung epithelial cell preparation, the cells were incubated with rat anti-CD32/CD16 and anti-CD45 for 30 min at 4°C, followed by incubation with anti-rat IgG micro beads and negative selection by Automacs. Cells were resuspended in DMEM containing 10% FBS, 1% streptomycin-penicillin and 10 μM β-mercaptoethanol and seeded into 48-well plate at 1×10^5/well for overnight culture followed by various treatments.

MOG immunization and induction of EAE

MOG immunization and EAE induction were performed as previously described[22,23]. In brief, 3-month wild-type and *Usp25*$^{-/-}$ littermates were immunized twice with 300 μg MOG_{35-55} peptide (MEVGWYRSPFSROVHLYRNGK) emulsified in CFA followed by intraperitoneal injection of pertussis toxin (100 μl) one day after immunization. The disease scores were assigned on a scale of 0–5 as follows: 0, none; 1, 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 taken from the EAE mice on day 12 after the second immunization and single cell suspension was prepared. An aliquot of these cells was taken and mRNA was isolated with Trizol reagent (Invitrogen). cDNA was synthesized and real-time PCR was performed.

Antibodies and reagent

Mouse control IgG (Santa Cruz Biotechnology, sc-2025) and rabbit control IgG (Millipore, 12-370), HRP-conjugated goat-anti mouse or rabbit IgG (Thermo Scientific, PA1-86717 and SA1-9510), HRP-conjugated anti-Myc (sc-40), mouse anti-Flag (Sigma, F1804), anti-Ubiquitin (sc-8017), anti-HA (Covance), anti-IκBα (sc-371), anti-TRAF5(sc-74503), Rabbit anti-K63-linked ubiquitin (Millipore, 05-1308), anti-ERK (sc-94), anti-JNK (sc-474), anti-p38 (sc-7149), anti-phospho-ERK(T202/Y204)(Cell Signaling Technology, 4370S), anti-phospho-JNK(T183/Y185) (CST, 9251S), anti-phospho-p38(T180/Y182) (CST, 4631L), anti-TRAF6 (sc-7221), anti-TRAF5 (sc-7220), anti-Act1 (sc-11444), and anti-β-Actin (Sigma, A2066) were purchased from the indicated manufactures. Mouse anti-TRAF6 was a generous gift from Dr. Hong-Bing Shu (Wuhan University, Wuhan, China)[19,32]. Rabbit anti-USP25 was described previously[24,47] and provided by Dr. Gemma Marfany (University de Barcelona, Barcelona, Spain). IL-1, TNF (Peprotech), IL-17 and IL-17F (BD Biosciences) were purchased from the indicated manufactures.

Constructs

Mammalian expression plasmids for Flag- or Myc-tagged USP25 and its truncated mutants were constructed in pcDNA6.0 vector. TRAF1-6 plasmids were kindly provided by Dr. Hong-Bing Shu (Wuhan University, China). Flag-USP25(C178S) was made with the site-directed mutagenesis kit (Stratagene, 200519). pcDNA-IL-17RA and IL-17RC were previously described[13]. HA-Ubiquitin (K48 only) and HA-Ubiquitin (K63 only) were kindly provided by Dr. Zhijian Chen (Southwestern Medical Center, Dallas).

Quantitative real-time PCR and ELISA

Cells treated with various stimuli were harvest in TriZol (Invitrogen, 15596-018) and the first-strand cDNA was synthesized with a reverse transcription kit (Invitrogen, 4368814). For KCΔ4 mRNA measurement in Tet-Off cells, the prepared mRNA was treated with RNase free DNase for 30 min followed by 65°C heating for 10 min and the oligo-dT and KCΔ4 RT primer (See **Supplementary Table**) was used for first-strand cDNA synthesis. Gene expression was examined with a Bio-Rad iCycler Optical system with an iQ SYBR Green Real-Time PCR kit (Bio-Rad Laboratories). Data were normalized to expression of β-Actin. Gene-specific primers were listed in Supplementary Table. TNF, IL-6 and CXCL1 (BD Biosciences) in samples were measured with ELISA kits from the indicated manufactures.

Coimmunoprecipitation, immunoblot and ubiquitination assays

Coimmunoprecipitation and immunoblot and ubiquitination assays were performed as previous described[48-50]. In brief, regular immunoprecipitation was performed in nature conditions in lysis buffer (20 mM Tris, pH7.4, 150 mM NaCl, 1mM EDTA, 20 mM NEM and 1% NP-40) and the immunoprecipitates were subjected to immunoblot analysis. For ubiquitination assay, the immunoprecipitates were re-extracted in lysis buffer containing 1% SDS and denatured by heating for 5 min. The supernatants were diluted with regular lysis buffer until the concentration of SDS was decreased to 0.1%, followed by reimmunoprecipitation with the indicated antibodies. The immunoprecipitates were analyzed

by immunoblotting with the ubiquitin or ubiquitin(K63-speccific) antibody. For in vitro ubiquitination experiments, proteins were expressed with a TNT Quick Coupled Transcription/Translation Systems kit (Promega, Madison, WI). Ubiquitination was analyzed with an ubiquitination kit (Enzo Life Sciences, Farmingdale, NY) following the protocols recommended by the manufacturer. Flag-USP25 and Flag-USP25(C178S) were purified by immunoprecipitation with anti-Flag gel (Sigma, A2220) from lysates of 293T cells transfected with Flag-USP25 or Flag-USP25(C178S) followed by elution with Flag peptide (50 μg/ml) (Sigma, F4799). In vitro deubiquitination assay was performed as previously described[36]. In brief, the in vitro ubiquitination reactions were terminated by EDTA (10 mM). The terminated reaction mixtures were incubated with Flag-tagged USP25 or USP25(C178S) at 37°C for 2 h and 16 °C for overnight before immunoblot analysis was performed.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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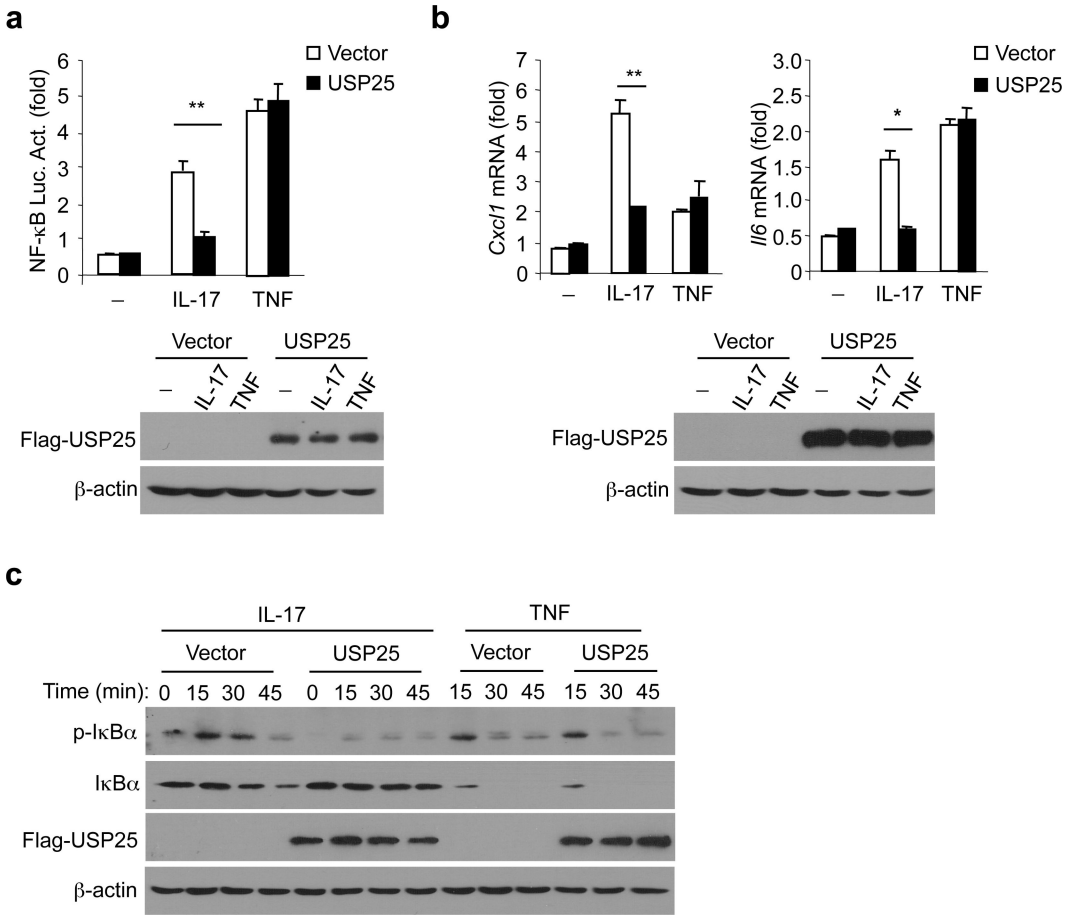

Figure 1. Overexpression of USP25 inhibits IL-17-triggered signaling

(**a**) Luciferase activity in HeLa cells transfected with NF-κB reporter plasmids, pRL-TK Renilla luciferase and Flag-USP25 or empty vector (Vector), followed by stimulation with IL-17 (50 ng/ml) or TNF (10 ng/ml) for 8 hours or no stimulation (-) 20 h after transfection. (**b**) Real-time RT-PCR analysis of IL-17-indued expression of *Cxcl1* and *Il6* mRNA in HeLa cells transfected with Flag-USP25 or empty vector (Vector) and stimulated with IL-17 (50 ng/ml) or TNF (10 ng/ml) for 2 h or left untreated (-) 20 h after transfection. (**c**) Immunoblot of phosphorylated and total IκBα in lysates from HeLa cells transfected with USP25 or empty vector followed by stimulation with IL-17 (50 ng/ml) or TNF (10 ng/ml) for 0-45 min. Data are representative of three independent experiments. Graphs show mean ± SD, n = 3. *p<0.05; **p<0.01.

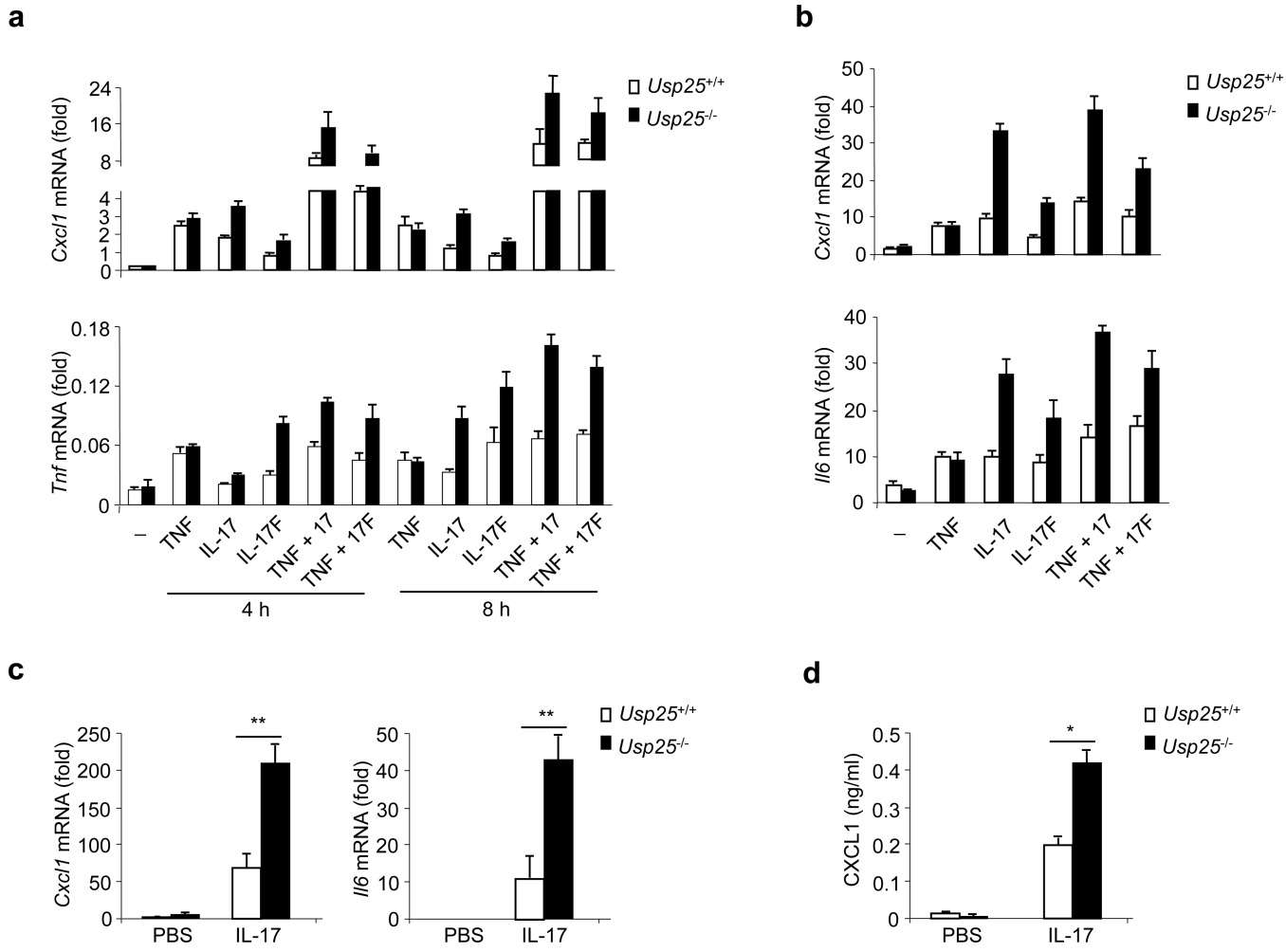

Figure 2. USP25 deficiency enhances IL-17-induced expression of pro-inflammatory cytokines

(**a**) Real-time RT-PCR analysis of *Cxcl1* and *Tnf* mRNA in wild-type and *Usp25*$^{-/-}$ MEFs left untreated (-) or stimulated by TNF (10 ng/ml), IL-17 (50 ng/ml), IL-17F (50 ng/ml) alone or with TNF plus IL-17 or TNF plus IL-17F for 4 or 8 h. (**b**) Real-time RT-PCR analysis of *Cxcl1* and *Il6* mRNA in wild-type and *Usp25*$^{-/-}$ primary lung epithelial cells left untreated (-) or stimulated by TNF (10 ng/ml), IL-17 (50 ng/ml), IL-17F (50 ng/ml) alone or with TNF plus IL-17 or TNF plus IL-17F for 2 h. (**c**) Real-time RT-PCR analysis of *Cxcl1* and *Il6* mRNA in peritoneal mesothelial cells isolated from wild-type and *Usp25*$^{-/-}$ mice intraperitoneally injected with PBS (100 μl) or IL-17 (0.5 μg in 100 μl PBS). (**d**) ELISA analysis of CXCL1 production in peritoneal lavage fluid isolated from wild-type and *Usp25*$^{-/-}$ mice treated as in (**c**). Data are representative of three independent experiments. Graphs show mean ± SD, n = 3. *p<0.01; **p<0.001.

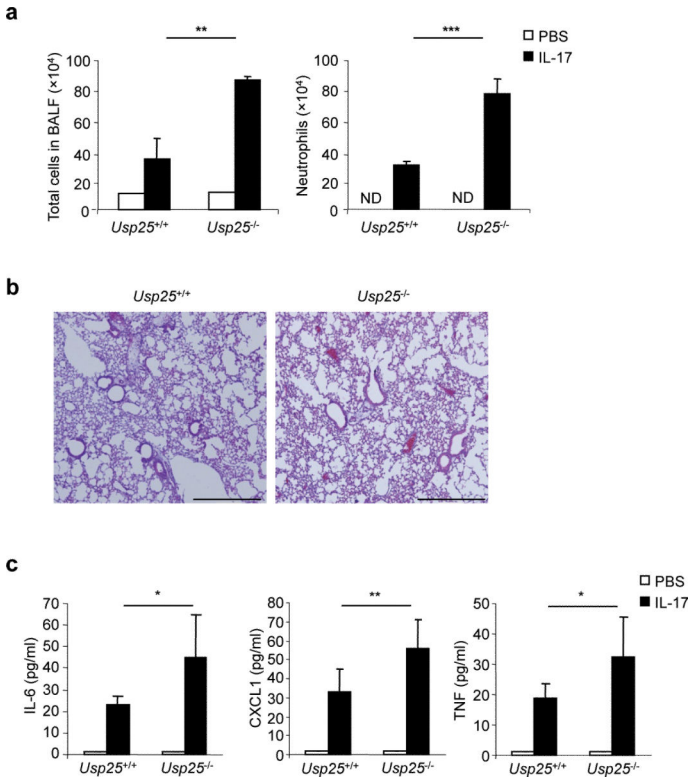

Figure 3. IL-17-induced pulmonary inflammation is enhanced in the absence of USP25

(**a**) Lung infiltrated total cells or neutrophils ($Gr1^+CD11b^+$) in bronchoalveolar lavage fluid (BALF) of wild-type and *Usp25*$^{-/-}$ mice (n=5) injected intranasally with PBS (50 μl) or IL-17 (1 μg in 50 μl PBS) 24 h after injection. ND, Not detected. (**b**) Histological comparison of lung tissues from mice treated as in (**a**). Lung sections were stained with hematoxylin and eosin. Original magnification, ×10; Scale bars, 500 μm. (**c**) ELISA analysis of CXCL1, IL-6 and TNF levels in BALF isolated from mice treated as in (**a**). Data shown are a representative of two independent experiments. Graphs show mean ± SD, n = 3. *p<0.05; **p<0.01; ***p<0.001.

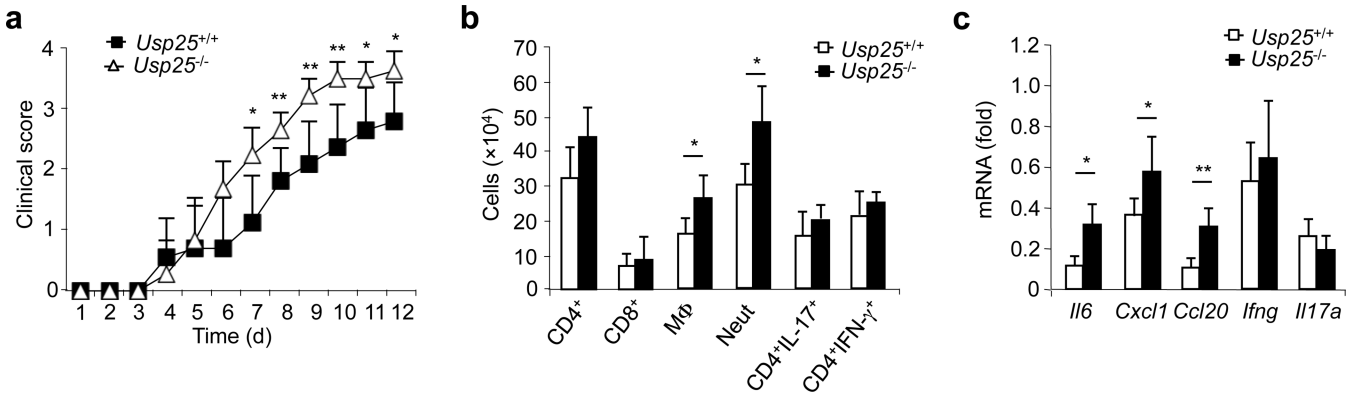

Figure 4. USP25 deficiency aggravates EAE severity

(**a**) Clinical scores (mean ± SD) of *Usp25*$^{-/-}$ mice and the wild-type littermates immunized twice with MOG_{35-55} were shown versus days after the second MOG immunization. (**b**) Flow cytometry analysis of infiltrated immune cells in the CNS of wild-type and *Usp25*$^{-/-}$ EAE mice at day 12 after second immunization and the absolute numbers of the immune cells were calculated. MΦ, macrophage; Neut, neutrophil. (**c**) Expression of genes in the CNS of wild-type and *Usp25*$^{-/-}$ EAE mice at day 12 after second immunization. Data are representative of three independent experiments. Graphs show mean ± SD, n = 5. *p<0.05; **p<0.01.

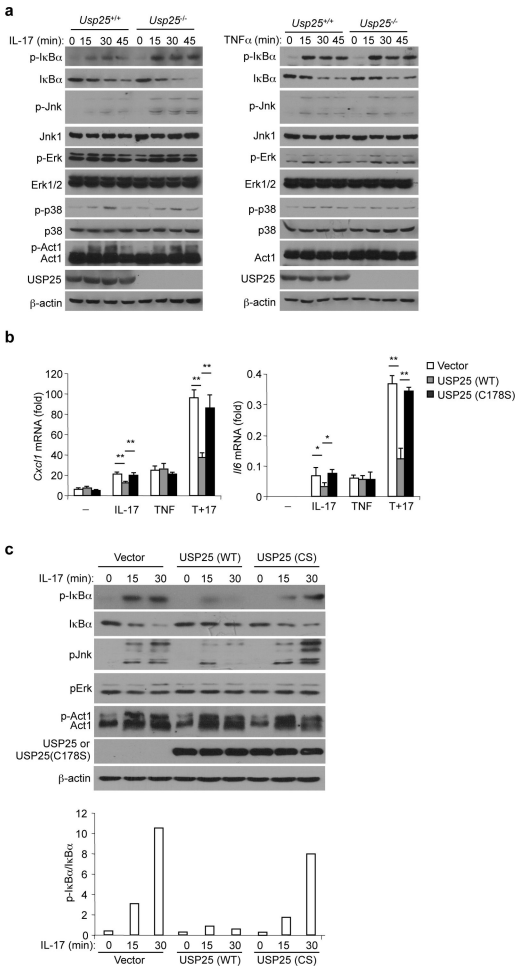

Figure 5. USP25 and its DUB activity are required for restriction of IL-17 signaling

(**a**) Immunoblot of IκBα, phosphorylated IκBα (p-IκBα), and phosphorylated MAPKs in lysates from wild-type and *Usp25*$^{-/-}$ MEFs stimulated with IL-17 (100 ng/ml) or TNF (10 ng/ml) for the indicated time points. (**b**) Real-time analysis of *Cxcl1* and *Il6* mRNA in wild-type and *Usp25*$^{-/-}$ MEFs reconstituted with empty vector (vector), USP25 or USP25(C178S) left untreated (-) or treated with IL-17 (50 ng/ml) or TNF (10 ng/ml) or IL-17 plus TNF. (**c**) Immunoblot of IκBα, phosphorylated IκBα (p-IκBα), and phosphorylated MAPKs in lysates from wild-type and *Usp25*$^{-/-}$ MEFs reconstituted with empty vector (vector), USP25 or USP25(C178S) treated with IL-17 (50 ng/ml) for the indicated time points. The intensity of p-IIκBα and IIκBα bands was quantified with the ImageJ program and the ratios of p-IIκBα/IIκBα were shown in the graph. Data are representative of three independent experiments. For **b**, graphs show mean ± SD, n = 3. *p<0.05; **p<0.01.

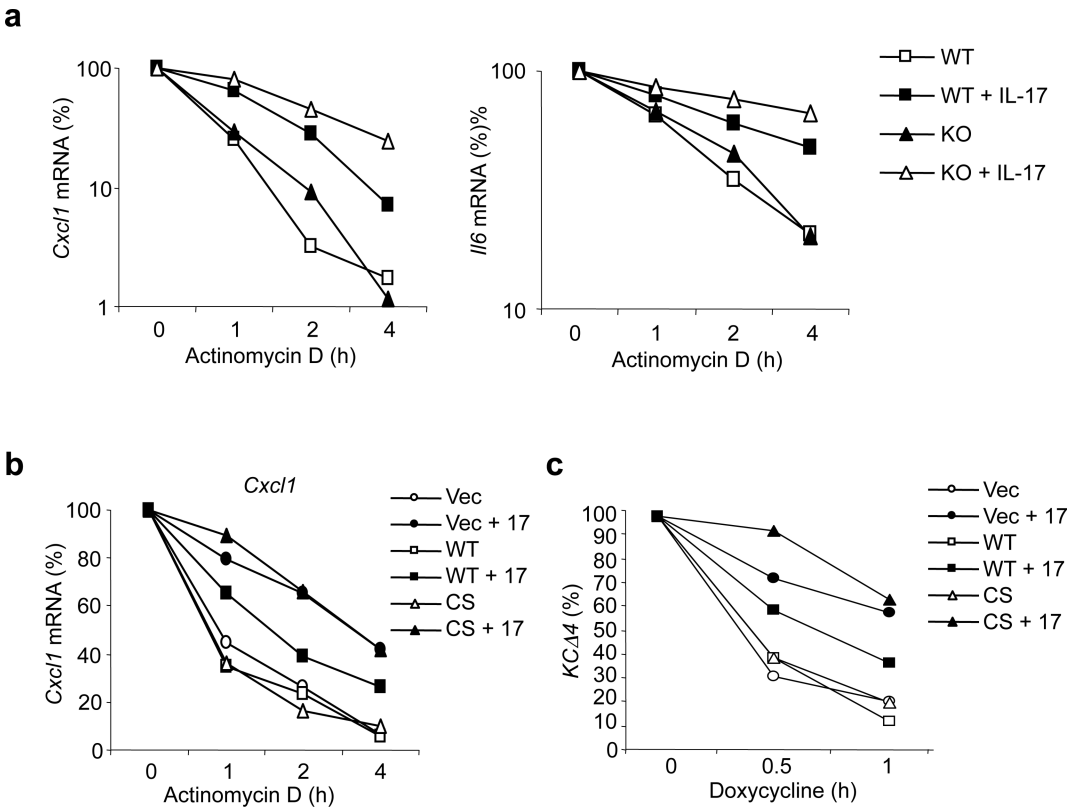

Figure 6. USP25 negatively regulates IL-17-mediated stabilization of chemokine mRNA

(**a**) Real-time RT-PCR analysis to measure *Cxcl1* and *Il6* mRNA levels in wild-type (WT) and USP25-deficient (KO) MEFs pretreated with TNF (10 ng/ml) for one hour followed by actinomycin D (5 μg/ml) or actinomycin D plus IL-17 (25 ng/ml) treatment for the indicated time points. (**b**) Real-time RT-PCR analysis to measure the *Cxcl1* mRNA levels in USP25-deficient (KO) MEFs reconstituted with vector, USP25 (WT) or USP25(C178S) (CS) pretreated with TNF (10 ng/ml) for one hour followed by actinomycin D (5 μg/ml) or actinomycin D plus IL-17 (25 ng/ml) treatment for the indicated time points. (**c**) Real-time RT-PCR analysis to measure the mRNA levels of *KCΔ4* in HeLa Tet-Off cells transfected with pTR2-KCΔ4 and vector, USP25(WT) or USP25(C178S) (CS) and then treated with doxycycline (1 μg/ml) or doxycycline plus IL-17 (25 ng/ml) for the indicated time points. Data shown are representative of three independent experiments.

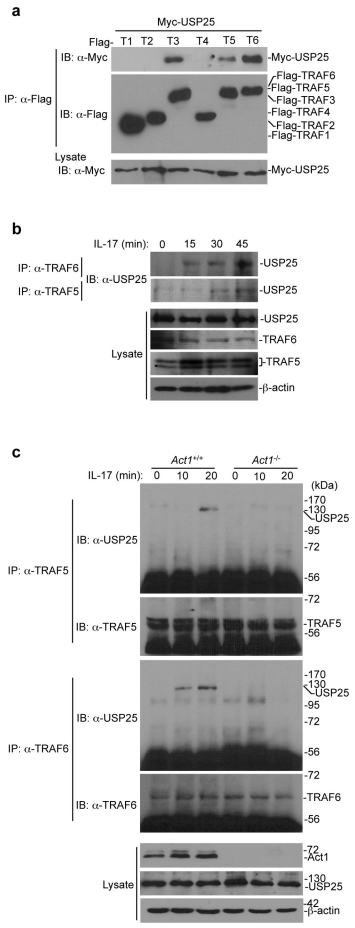

Figure 7. IL-17 induces USP25-TRAF5 and USP25-TRAF6 association

(**a**) Immunoassay of 293T cells transfected with Myc-USP25 and Flag-tagged TRAF1-6 (T1-T6), followed by immunoprecipitation (IP) with anti-Flag and immunoblot analysis with anti-Myc or anti-Flag. Lysate, immunoblot analysis of whole-cell lysates without immunoprecipitation (throughout). (**b**) Immunoassay of lysates of human bronchial epithelial cells (HBECs) treated with hIL-17 (100 ng/ml) for various time, followed by immunoprecipitation with anti-TRAF5 or anti-TRAF6 and immunoblot analysis with anti-USP25 (top two panels). The expression levels of the indicated proteins in the lysates were examined by immunoblot (bottom four panels). (**c**) Immunoassay of lysates from wild-type or *Act1*$^{-/-}$ MEFs treated with hIL-17 (100 ng/ml) for various time points, followed by immunoprecipitation with anti-TRAF5 or anti-TRAF6 and immunoblot analysis with anti-USP25, anti-TRAF5 or anti-TRAF6. Data are a representative of two (**a**) or three (**b,c**) independent experiments.

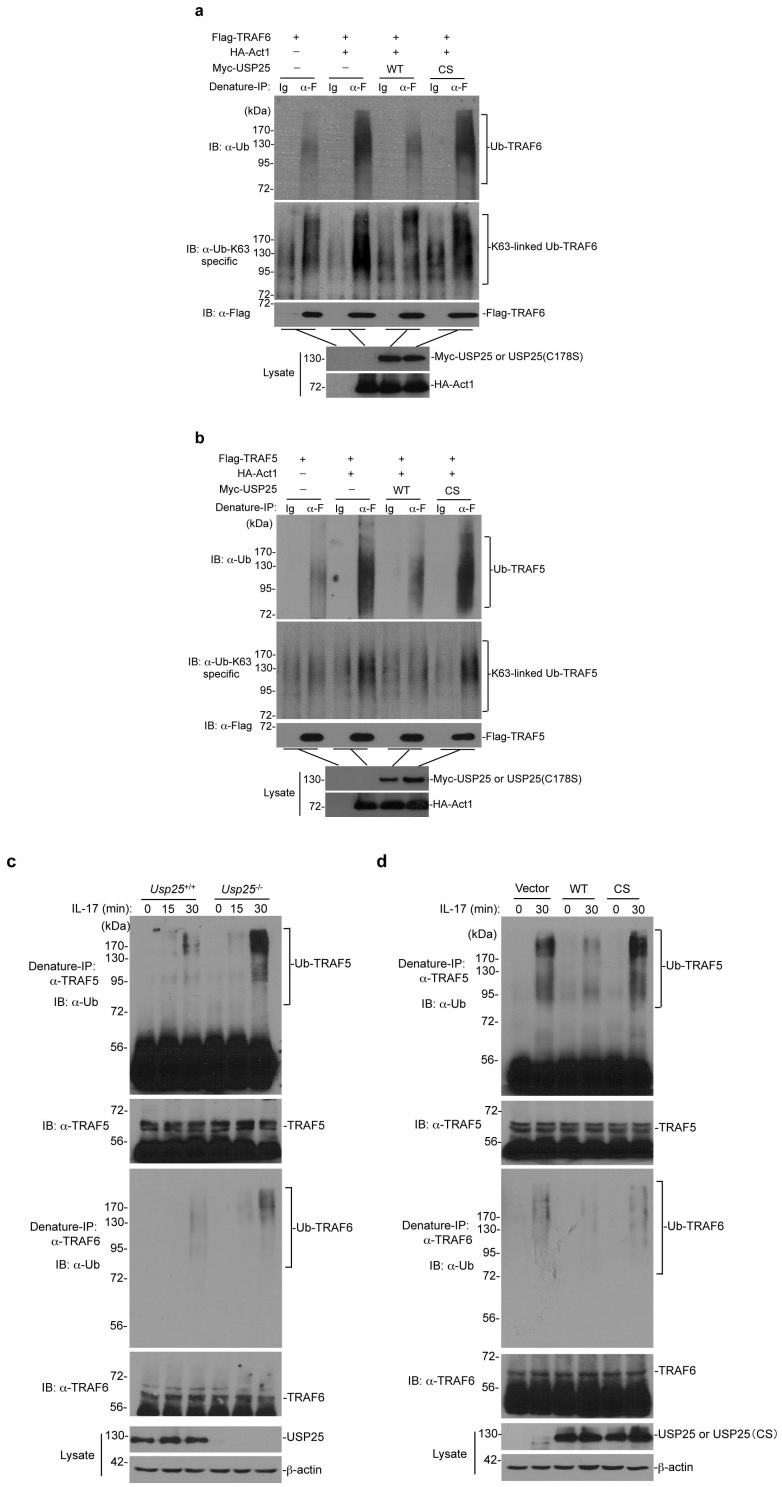

Figure 8. USP25 deubiquitinates Act1-mediated K63-linked ubiquitination of TRAF6 and TRAF5

(**a,b**) Immunoassay of 293T cells transfected with the indicated plasmids, followed by denature-immunoprecipitation (Denature-IP) with anti-Flag and immunoblot analysis with anti-ubiquitin (Ub), anti-ubiquitin(K63 specific) or anti-Flag 20 h after transfection. The

expression levels of USP25, USP25(C178S) or Act1 were analyzed by immunoblot with anti-Myc or anti-HA. (**c**) Immunoassay of lysates of wild-type and *Usp25*$^{-/-}$ MEFs treated with IL17 (200 ng/ml) for the indicated time points, followed by denature-IP with anti-TRAF5 or anti-TRAF6 and immunoblot with anti-ubiquitin, anti-TRAF5 or anti-TRAF6. (**d**) Immunoassay of lysates of *Usp25*$^{-/-}$MEFs reconstituted with empty vector (Vector), USP25 (WT) or USP25(C178S) (CS) treated with IL17 (200 ng/ml) for 30 min, followed by denature-IP with anti-TRAF5 or anti-TRAF6 and immunoblot with anti-ubiquitin, anti-TRAF5 or anti-TRAF6. The expression level of reconstituted proteins were analyzed by immunoblot with anti-USP25. Data are representative of three independent experiments.