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The ubiquitin-specific protease USP8 is critical for the development and homeostasis of T cells

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The modification of proteins by ubiquitin has a major role in cells of the immune system and is counteracted by various deubiquitinating enzymes (DUBs) with poorly defined functions. Here we identified the ubiquitin-specific protease USP8 as a regulatory component of the T cell antigen receptor (TCR) signalosome that interacted with the adaptor Gads and the regulatory molecule 14-3-3β. Caspase-dependent processing of USP8 occurred after stimulation of the TCR. T cell–specific deletion of USP8 in mice revealed that USP8 was essential for thymocyte maturation and upregulation of the gene encoding the cytokine receptor IL-7Rα mediated by the transcription factor Foxo1. Mice with T cell–specific USP8 deficiency developed colitis that was promoted by disturbed T cell homeostasis, a predominance of CD8$^+$ γδ T cells in the intestine and impaired regulatory T cell function. Collectively, our data reveal an unexpected role for USP8 as an immunomodulatory DUB in T cells.

Ubiquitination has a fundamental role in the immune system, but the physiological relevance of the more than 90 mammalian deubiquitinating enzymes (DUBs) is poorly defined[1,2]. The ubiquitin-specific protease USP8 (UBPY) participates in the endosomal sorting of transmembrane proteins[3,4] and interacts with the proline-rich SH3 domain of the signal-transducing adaptor STAM2, which is a component of the endosomal sorting complex ESCRT-0[5]. USP8 has been shown to deubiquitinate both cargo proteins and ESCRT-0 proteins and to thus modulate their function and stability[3,4]. Proteolytic cleavage of human USP8 increases its enzymatic activity, and somatic mutations that promote the processing of USP8 can cause Cushing's disease by stabilizing epidermal growth factor receptor[6].

USP8 contains two atypical SH3-binding motifs (SH3BMs) that flank a binding motif (14-3-3BM) for members of the 14-3-3 family of regulatory proteins, which inhibit USP8 activity[5–8]. Peptide-binding studies have revealed that the amino-terminal SH3BM of USP8 exhibits a higher binding affinity for an SH3 domain of Gads, a signaling adaptor downstream of the T cell antigen receptor (TCR), than that of the carboxy-terminal SH3BM of USP8 for the STAM2 SH3 domain[9,10]. Gads has high expression in T cells and modulates signal diversification[11]. Gads forms a constitutive adaptor complex with the SH2 domain–containing adaptor SLP-76. Upon activation of the TCR, Gads also associates with the scaffolding protein Lat. However, whether Gads interacts with USP8 *in vivo* remains unresolved. Furthermore, the function of USP8 in T cells remains undefined.

Several ubiquitin E3 ligases, including Cbl-b, Itch, GRAIL and roquin, are essential for the induction of anergy in T cells. As a consequence, loss of these proteins is associated with autoimmunity[1,12]. Likewise, several DUBs, including A20, USP9X, USP15 and CYLD, are essential for proper T cell function[1,2,13,14]. Overexpression of USP8 leads to the formation of a complex with GRAIL and the DUB otubain1. It has been suggested that an inactive isoform of otubain1 promotes USP8-mediated deubiquitinylation and stabilization of GRAIL[15].

In addition to being controlled by TCR-mediated signals, the function and development of T cells is controlled by cytokines and chemokines, which transmit signals through their cognate receptors. A tightly regulated key participant in the development and function of lymphocytes is the interleukin 7 receptor (IL-7R), whose expression is transiently downregulated on CD4$^+$CD8$^+$ double-positive (DP) thymocytes and is re-expressed by CD4$^+$ or CD8$^+$ single-positive (SP) thymocytes. In the periphery, naive T cells have high expression of IL-7R[16]. The transcription factor Foxo1 positively controls expression

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of the gene encoding IL-7R (*Il7r*; called '*Il7ra*' here)[17,18] and affects the expression of genes encoding the transcription factor KLF2, the chemokine receptor CCR7 and the lymph node–homing receptor CD62L[17,19]. Furthermore, Foxo1-regulated transcription programs are essential for the function of regulatory T cells (T_{reg} cells)[20]. Foxo1 itself is regulated by post-translational modifications and sequestration by proteins of the 14-3-3 family[21,22].

Defects in TCR signaling or thymocyte development often cause chronic lymphopenia, which results in a proliferative response and the conversion of T cells from a naive phenotype to an effector phenotype[19,23]. IL-7R signaling and complexes of self peptide and major histocompatibility complex (MHC) are vital for lymphopenia-induced proliferation and for the homeostasis of naive T cells[19,23]. Furthermore, altered T cell homeostasis predisposes organisms to the development of colitis typically promoted by defective T_{reg} cell function[18,24].

Here we found that USP8 interacted with Gads and 14-3-3β and that it was processed after activation via the TCR in a caspase-dependent manner. USP8 was essential for normal development and homeostasis of T cells by securing maturation and Foxo1-mediated upregulation of *Il7ra*. Concordantly, T cell–specific inactivation of USP8 resulted in colitis associated with a lack of suppressive effect of T_{reg} cells on the considerably abundant $CD8^+$ γδT cells in inflamed colons. Our data establish USP8 as a distinct DUB with roles in the development and homeostasis of T cells.

RESULTS

Proteolytic processing of USP8 and binding to Gads

To identify potential USP8-interacting proteins in T cells, we performed a yeast two-hybrid screen with fragments of USP8 as the 'bait' and a thymocyte-derived cDNA library. Screening with bait 1, which contained the amino-terminal SH3BM of USP8, yielded Gads as a binding partner (**Supplementary Fig. 1**). Furthermore, we detected binding of 14-3-3β with bait 2, which encompassed the 14-3-3BM of USP8.

We confirmed those interactions by coimmunoprecipitation experiments with affinity-tagged proteins. To analyze the contribution of the USP8 SH3BMs to the interaction with Gads, we replaced critical proline residues with alanine (P405A or P738A or both (P405A,P738A)). Replacement of the amino-terminal proline (P405A) resulted in significantly diminished binding of USP8 to Gads (**Fig. 1a**). The interaction with 14-3-3 proteins depends on phosphorylation of a serine residue within the binding motif. Replacement of this serine in the 14-3-3BM of USP8 (S718A)[7] abolished the interaction of these proteins (**Fig. 1b**), which indicated that USP8 bound to 14-3-3β in a manner dependent on phosphorylated Ser718. We confirmed the interaction of endogenous Gads and USP8 in T cells by immunoprecipitation of endogenous USP8 (**Fig. 1c**). Furthermore, endogenous 14-3-3β interacted with FLAG-tagged wild-type USP8, but not with USP8(S718A), in HEK293T human embryonic kidney cells (**Fig. 1d**).

Individual TCRs are hypothesized to form nanoclusters in the absence of stimulation and to form microclusters upon activation[25]. As Gads and proteins of the 14-3-3 family represent proximal components of TCR signaling[11,26], USP8 might also participate in this. We therefore grew $CD4^+$ T cells *ex vivo* and restimulated the cells with beads coupled to the antibodies anti-CD3 and anti-CD28, which simultaneously trigger signaling via the TCR and costimulatory molecules and allow the precipitation of proteins associated with the TCR and the coreceptor CD28. We found that USP8 and Gads bound to the TCR-CD28 cluster and accumulated together upon stimulation (**Fig. 1e**). After stimulation of the TCR, USP8 was proteolytically processed, which yielded an amino-terminal 70-kilodalton cleavage product (**Fig. 1f**). Cleavage was inhibited by pretreatment with the 'pan-caspase' inhibitor z-VAD (**Fig. 1g**), which indicated that USP8 was regulated in a caspase-dependent manner.

To evaluate whether Gads and 14-3-3β are modified by ubiquitin and might represent substrates for USP8-mediated deubiquitination, we coexpressed these proteins with wild-type USP8 or the USP8(C786A) variant lacking deubiquitinating activity. Consistent with ubiquitin modification, we detected bands of higher molecular mass than that of unmodified Gads and 14-3-3β upon coexpression and immunoprecipitation with USP8(C786A), but not upon coexpression and immunoprecipitation with wild-type USP8 (**Fig. 1h**). We confirmed USP8-mediated modulation of Gads and ubiquitination of 14-3-3β by coexpression of FLAG-tagged ubiquitin (**Supplementary Fig. 2a**). Furthermore, we identified ubiquitination of 14-3-3β at Lys9 and Lys189 by mass spectrometry (**Supplementary Fig. 2b**). In summary, our results showed that USP8 interacted with the TCR signalosome and bound Gads and 14-3-3, whereas USP8 was a target of caspase-dependent regulation.

Requirement for USP8 in T cell development

To analyze the physiological role of USP8 in T cells, we mated mice with *lox*P-flanked alleles ($Usp8^{f/f}$)[3] to mice with transgenic expression of Cre recombinase from the T cell–specific *Cd4* promoter (*Cd4*-Cre), to generate $Usp8^{f/f}$*Cd4*-Cre progeny. We confirmed the deletion of *Usp8* in DP and $CD4^+$ SP (SP4) $Usp8^{f/f}$*Cd4*-Cre thymocytes by Southern blot and/or immunoblot analysis (**Fig. 2a** and **Supplementary Fig. 3a,b**). However, $CD8^+$ SP (SP8) $Usp8^{f/f}$*Cd4*-Cre thymocytes showed no reduction in USP8 expression relative to its expression in $Usp8^{f/f}$ cells (**Supplementary Fig. 3b**), presumably due to an advantage of 'escapees' that reach an early developmental stage without losing expression of USP8. $Usp8^{f/f}$*Cd4*-Cre mice exhibited a lower frequency of mature SP4 and SP8 thymocytes that resulted in a 2- to 3.5 fold lower total number relative to that in control ($Usp8^{f/f}$ or $Usp8^{+/+}$*Cd4*-Cre) mice, whereas total number of DP thymocytes was unaltered (**Fig. 2b**). The reduction in SP8 thymocytes was even more pronounced within the TCR^{hi} subpopulation (**Fig. 2b**). Accordingly, medullary regions were more sparsely distributed and smaller in the thymi of $Usp8^{f/f}$*Cd4*-Cre mice than in those of $Usp8^{f/f}$ mice (**Fig. 2c**). Furthermore, the frequency of thymic $CD25^+Foxp3^+$ T_{reg} cells among the $CD4^+$ thymocyte population and the thymic $NK1.1^+$ natural killer T cell population was diminished in $Usp8^{f/f}$*Cd4*-Cre mice relative to their frequency in $Usp8^{f/f}$ mice (**Fig. 2d**). To characterize $CD4^-CD8^-$ double negative (DN), DP, SP4 and SP8 thymocytes, we analyzed developmental markers. The expression of TCRβ by $Usp8^{f/f}$*Cd4*-Cre SP4 and SP8 cells was diminished (**Fig. 2e**). While CD62L expression was lower in $Usp8^{f/f}$*Cd4*-Cre SP4 cells than in $Usp8^{f/f}$ SP4 cells, a lower frequency of $Usp8^{f/f}$*Cd4*-Cre SP8 cells than $Usp8^{f/f}$ SP4 cells expressed the negative regulator CD5 (**Fig. 2e**). We did not observe downregulation of expression of the surface marker CD24, which also correlates with progressed maturation, in $Usp8^{f/f}$*Cd4*-Cre SP4 and SP8 cells relative to its expression in their $Usp8^{f/f}$ counterparts (**Fig. 2e**). A greater frequency of $Usp8^{f/f}$*Cd4*-Cre SP4 cells than $Usp8^{f/f}$ SP4 cells had high expression of the activation marker CD69 (**Fig. 2e**), which indicated that more cells were engaged in early acting interactions of the TCR and peptide-MHC complexes. Likewise, we found that in $Usp8^{f/f}$*Lck*-Cre mice ($Usp8^{f/f}$ mice that express Cre from the T cell–specific *Lck* promoter), the pattern of USP8 depletion was similar to that in $Usp8^{f/f}$*Cd4*-Cre mice and did not reveal a defect in the development of DN cells (**Supplementary Fig. 3c,d**). However, we observed a lower frequency of SP4 and SP8 thymocyte populations in

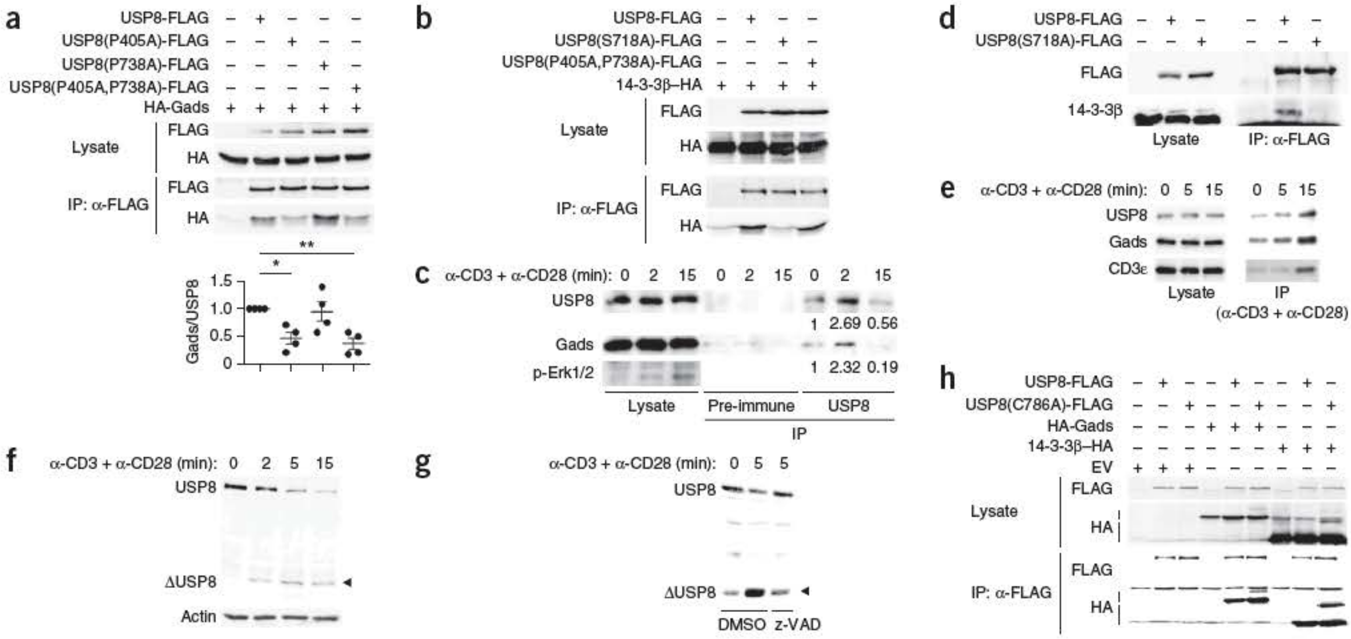

Figure 1 USP8 interacts with Gads, 14-3-3β and the TCR signalosome. (**a**,**b**) Immunoprecipitation (IP), with anti-FLAG (α-FLAG), and immunoblot analysis of the precipitation of Gads tagged with hemagglutinin at the amino terminus (HA-Gads) (**a**) or 14-3-3β tagged with hemagglutinin at the carboxyl terminus (14-3-3β–HA) (**b**), versus USP8 tagged with FLAG at the carboxyl terminus, in HEK293T cells transfected with various combinations of tagged USP8, Gads and 14-3-3β expression vectors (above blots); Lysate (upper blots), immunoblot analysis without immunoprecipitation (throughout). Bottom (**a**), quantification of precipitated Gads relative to USP8, determined by densitometry and normalized to the ratio of Gads precipitated by wild-type USP8 (columns correspond to lanes above). Each symbol represents an individual sample; small horizontal lines indicate the mean (±s.e.m.). (**c**) Direct immunoprecipitation (with anti-USP8 or preimmune serum) and immunoblot analysis of the interaction of endogenous USP8 and Gads and the phosphorylation (p-) of Erk1/2 in lysates of wild-type splenic $CD4^+$ T cells that underwent population expansion for 4 d, then were starved by depletion of serum and agonists and then restimulated for various times (above lanes) with anti-CD3 and anti-CD28. Numbers below lanes indicate abundance of proteins in the immunoprecipitate relative to that in unstimulated control cells, determined by densitometry. (**d**) Immunoprecipitation (with anti-FLAG) and immunoblot analysis (with anti-FLAG and anti-14-3-3β) of HEK293T cells transfected with FLAG-tagged USP8 expression vectors (above lanes). (**e**) Immunoblot analysis of the interaction of USP8 with TCR-CD28 complexes in $CD4^+$ T cells that underwent population expansion for 4 d, followed by restimulation (immunoprecipitation) for various times (above lanes) with anti-CD3 and anti-CD28 coupled to superparamagnetic beads, and purification of protein complexes bound to CD3 and CD28. (**f**) Immunoblot analysis of USP8 processing in $CD4^+$ T cells that underwent population expansion and restimulation as in **c**; arrowhead (right margin) indicates the cleavage product of USP8 (ΔUSP8). Actin (bottom blot) serves as a loading control throughout. (**g**) Immunoblot analysis of USP8 processing (as in **f**) in $CD4^+$ T cells treated with the vehicle DMSO (dimethyl sulfoxide) or with z-VAD and stimulated for various times (above lanes) with anti-CD3 and anti-CD28. (**h**) Immunoprecipitation and immunoblot analysis (as in **a**) of the effect of USP8 on the ubiquitination of Gads and 14-3-3β in HEK293T cells transfected with various combinations (above lanes) of expression vector for FLAG-tagged wild-type or mutant USP8, plus empty vector (EV) or a Gads or 14-3-3β expression vector; vertical bars along left margin indicate mobility shifts due to ubiquitin modification. $*P < 0.01$ and $**P < 0.001$ (unpaired, two-sided t-test). Data are representative of (**a**, top, **b**) or from (**a**, bottom) four independent experiments (**a**,**b**) or are representative of at least three independent experiments (**b**–**h**).

Usp8$^{f/f}$*Lck*-Cre mice than in *Usp8*$^{f/f}$ mice, and TCRβ expression in in *Usp8*$^{f/f}$*Lck*-Cre mice was similar to that achieved by deletion mediated by *Cd4*-Cre (**Supplementary Fig. 3e**). Together these results showed that USP8 was critical for thymocyte maturation.

Requirement for USP8 in proliferation

Ex vivo, lack of USP8 compromised thymocyte proliferation upon stimulation with anti-CD3 in the presence or absence of costimulation with IL-2 or anti-CD28 (**Fig. 3a**). We also observed defects in the proliferation of cells stimulated with the phorbol ester PMA plus ionomycin, which bypasses proximal TCR signaling (**Fig. 3a**); this indicated that lack of USP8 also affected downstream processes. Lack of proliferation was accompanied by diminished TCRβ expression (**Fig. 3a**). We did not detect differences in the apoptosis of *Usp8*$^{f/f}$*Cd4*-Cre thymocyte relative to that of *Usp8*$^{f/f}$ thymocyte (**Supplementary Fig. 3f**). Moreover, *Usp8*$^{f/f}$*Cd4*-Cre thymocytes stimulated with anti-CD3 were unable to secrete IL-2 (**Fig. 3b**).

Qualitative alterations of T cell signaling influence thymocyte selection. For example, Gads-null mice display defects in negative selection[27]. Thus, we crossed *Usp8*$^{f/f}$*Cd4*-Cre mice to HY mice, which have transgenic expression of a TCR that recognizes the male-specific HY antigen (called 'HY-TCR' here). As in *Usp8*$^{f/f}$*Cd4*-Cre mice, the frequency of mature HY-TCR^+ SP4 or SP8 thymocytes was lower in female *Usp8*$^{f/f}$*Cd4*-Cre HY-TCR^+ mice than in their *Usp8*$^{f/f}$ HY-TCR^+ counterparts (**Fig. 3c**, top). However, negative selection was not compromised in male *Usp8*$^{f/f}$*Cd4*-Cre HY-TCR^+ mice (**Fig. 3c**, bottom). These results suggested that USP8 was dispensable for negative selection but was required for thymocyte proliferation.

USP8 deficiency affects IL-7Rα expression and Foxo1 activity

Usp8$^{f/f}$*Cd4*-Cre thymocytes did not display differences in the stability of STAM2 or overall ubiquitination relative to that of *Usp8*$^{f/f}$ thymocytes (**Supplementary Fig. 4a,b**). Moreover, endocytosis of the TCR upon stimulation was unaffected by the loss of USP8 (**Supplementary Fig. 4c**). These results suggested that the function of USP8 in T cells differed from the ESCRT-associated role in mouse embryonic fibroblasts and hepatocytes[3]. The activation of mitogen-activated protein kinases and the kinase Akt, the phosphorylation

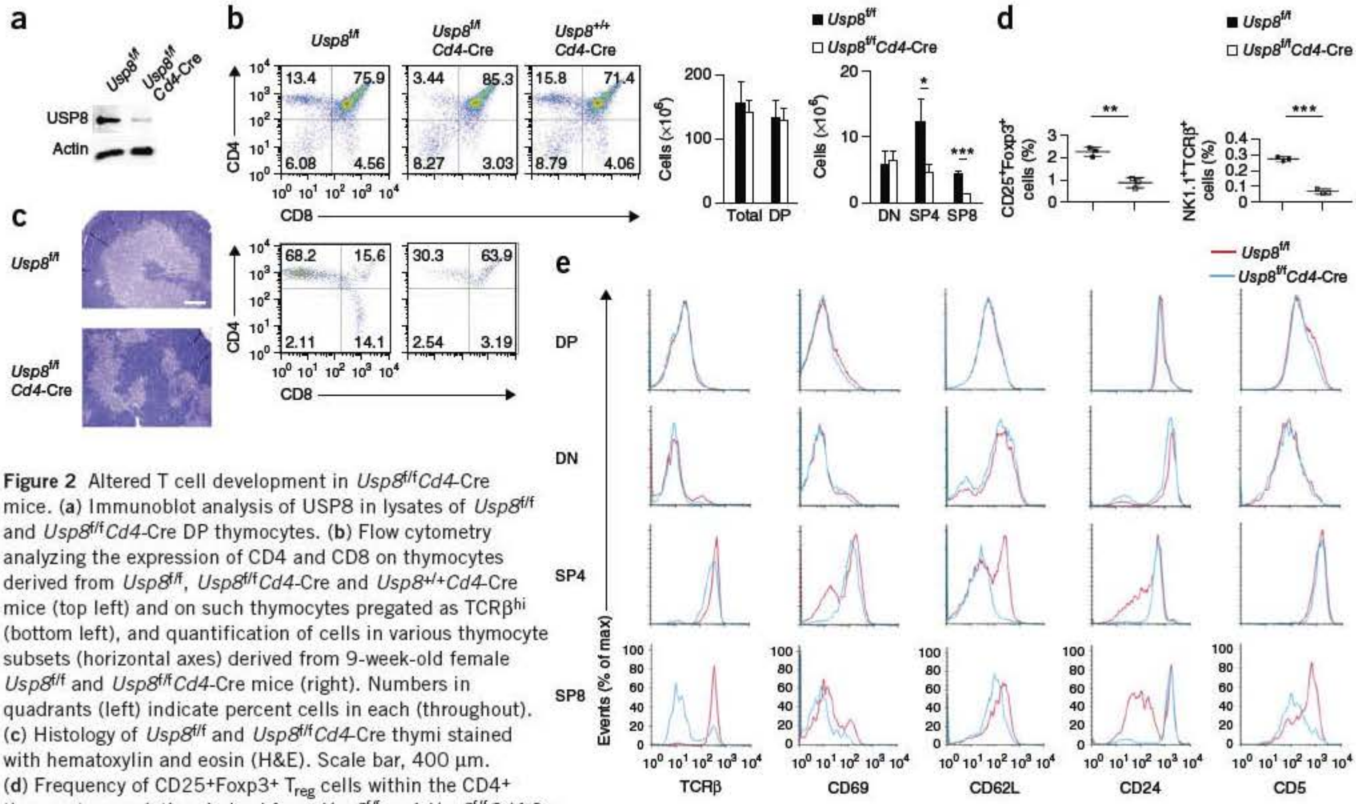

Figure 2 Altered T cell development in *Usp8*$^{f/f}$*Cd4*-Cre mice. (**a**) Immunoblot analysis of USP8 in lysates of *Usp8*$^{f/f}$ and *Usp8*$^{f/f}$*Cd4*-Cre DP thymocytes. (**b**) Flow cytometry analyzing the expression of CD4 and CD8 on thymocytes derived from *Usp8*$^{f/f}$, *Usp8*$^{f/f}$*Cd4*-Cre and *Usp8*$^{+/+}$*Cd4*-Cre mice (top left) and on such thymocytes pregated as TCRβ^{hi} (bottom left), and quantification of cells in various thymocyte subsets (horizontal axes) derived from 9-week-old female *Usp8*$^{f/f}$ and *Usp8*$^{f/f}$*Cd4*-Cre mice (right). Numbers in quadrants (left) indicate percent cells in each (throughout). (**c**) Histology of *Usp8*$^{f/f}$ and *Usp8*$^{f/f}$*Cd4*-Cre thymi stained with hematoxylin and eosin (H&E). Scale bar, 400 μm. (**d**) Frequency of CD25+Foxp3+ T_{reg} cells within the CD4+ thymocyte population derived from *Usp8*$^{f/f}$ and *Usp8*$^{f/f}$*Cd4*-Cre mice (left), and flow cytometry of thymic NK1.1+ TCRβ+ natural killer T (NKT) cells (right). Each symbol represents an individual mouse; small horizontal lines indicate the mean (±s.d.). (**e**) Flow cytometry analyzing the surface markers TCRβ, CD69, CD62L, CD24 and CD5 on thymocyte subsets derived from *Usp8*$^{f/f}$ and *Usp8*$^{f/f}$*Cd4*-Cre mice. **P* < 0.05, ***P* < 0.01 and ****P* < 0.001 (unpaired, two-sided *t*-test). Data are representative of three independent experiments (**a–d**; mean and s.e.m. in **b**) or at least six independent experiments (**e**).

and degradation of the inhibitor IκB in response to TCR stimulation, the stability of 14-3-3β or Gads, binding of the transcription factors NF-κB and AP-1 to DNA, overall tyrosine phosphorylation, and calcium influx were not altered in *Usp8*$^{f/f}$*Cd4*-Cre thymocytes (**Supplementary Fig. 4d–j**). These results suggested that USP8 was dispensable for canonical TCR signaling.

To detect the fundamental cause of the observed alterations in USP8-deficient thymocytes, we analyzed the RNA expression of DP thymocytes by microarray. Around 74 genes were dysregulated more than twofold in *Usp8*$^{f/f}$*Cd4*-Cre thymocytes relative to their regulation in *Usp8*$^{f/f}$ cells (**Supplementary Fig. 5a,b**). *Il7ra* mRNA showed the greatest downregulation in the absence of USP8 (**Supplementary Fig. 5a,b**). Concordantly, upregulation of the surface expression of IL-7Rα was impaired on *Usp8*$^{f/f}$*Cd4*-Cre SP thymocytes relative to its upregulation on *Usp8*$^{f/f}$ SP thymocytes (**Fig. 4a**). Likewise, at the preceding DP stage of development, the abundance of *Il7ra* mRNA was lower in *Usp8*$^{f/f}$*Cd4*-Cre thymocytes than in *Usp8*$^{f/f}$ thymocytes (**Fig. 4a**). Similarly, less mRNA encoding CCR7 (*Ccr7*) and cyclin D2 (*Ccnd2*) was present in *Usp8*$^{f/f}$*Cd4*-Cre DP thymocytes than in *Usp8*$^{f/f}$ DP thymocytes, and upregulation of the expression of CCR7 protein was blocked in SP4 thymocytes (**Fig. 4a**). *Il7ra*, *Ccr7* and *Sell* (which encodes CD62L) constitute direct or indirect targets of Foxo1 (refs. 17,19), which is regulated by proteins of the 14-3-3 family[21,22]. Whereas the abundance of Foxo1 protein and its relocalization in response to stimulation of the TCR were unaltered in *Usp8*$^{f/f}$*Cd4*-Cre thymocytes relative to that in *Usp8*$^{f/f}$ thymocytes (**Fig. 4b** and data not shown), binding of Foxo1 to the *Il7ra* promoter was severely compromised in *Usp8*$^{f/f}$*Cd4*-Cre CD4$^+$thymocytes relative to such binding in *Usp8*$^{f/f}$ CD4$^+$ thymocytes (**Fig. 4c**). These results indicated that USP8 directly or indirectly affected thymocyte development via the Foxo1–IL-7Rα axis.

Critical role for USP8 in T cell homeostasis

Consistent with the defect in thymocyte development, the frequency of SP4 and SP8 T cells and total number of T cells was much lower in the spleen and mesenteric lymph nodes (MLNs) of *Usp8*$^{f/f}$*Cd4*-Cre mice than in that of *Usp8*$^{f/f}$ or *Usp8*$^{+/+}$*Cd4*-Cre mice (**Fig. 5a**, **Supplementary Fig. 6a** and data not shown). *Usp8*$^{f/f}$*Cd4*-Cre mice had fewer naive CD4$^+$ T cells in the periphery and a greater frequency of CD44^{hi} effector (CD62L^{lo}) and memory (CD62L^{hi}) T cells relative to that of *Usp8*$^{f/f}$ or *Usp8*$^{+/+}$*Cd4*-Cre mice (**Fig. 5a** and **Supplementary Fig. 6a**). Deletion of *Usp8* was incomplete in *Usp8*$^{f/f}$*Cd4*-Cre peripheral T cells (**Supplementary Fig. 6b,c**), which indicated that cells that failed to delete *Usp8* exhibited an advantage over their *Usp8*-deficient counterparts in the periphery. IL-7Rα expression was lower in the few remaining *Usp8*$^{f/f}$*Cd4*-Cre naive T cells than in their *Usp8*$^{f/f}$ counterparts, but was not lower in *Usp8*$^{f/f}$*Cd4*-Cre effector T cells than in *Usp8*$^{f/f}$ effector T cells (**Supplementary Fig. 6d**). Likewise, *Lck*-Cre–mediated deletion of *Usp8* caused a substantial reduction in the frequency of T cells in the periphery, pronounced diminution of the naive T cell population and a decrease in IL-7Rα expression on naive T cells (**Supplementary Fig. 6e**). CCR7-expressing CD4$^+$ cells were also almost completely absent in the periphery of *Usp8*$^{f/f}$*Cd4*-Cre mice (**Supplementary Fig. 6f**). We observed no significant difference in the frequency of T_{reg} cells (among CD4$^+$ cells), granulocytes, monocytes,

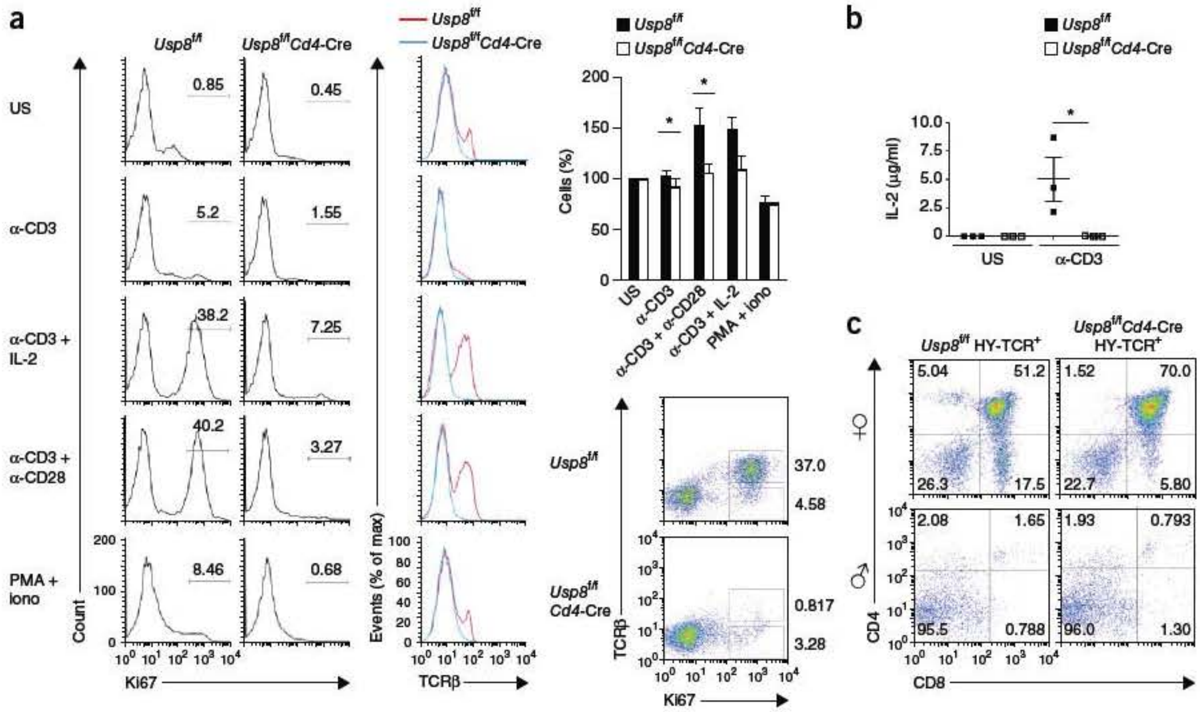

Figure 3 Diminished thymocyte proliferation after depletion of USP8. (**a**) Flow cytometry analyzing the expression of Ki67 and TCRβ (left) by *Usp8*$^{f/f}$ and *Usp8*$^{f/f}$*Cd4*-Cre thymocytes left unstimulated (US) or stimulated for 72 h with plate-bound anti-CD3 alone or in the presence of anti-CD28 or IL-2, or with PMA plus ionomycin (PMA + iono) (left), and quantification of thymocytes in parallel with those analyses, presented relative to the abundance of unstimulated cells (top right). Numbers above bracketed lines (left) indicate percent Ki67$^+$ cells. Bottom right, correlation of the expression of TCRβ and Ki67 by *Usp8*$^{f/f}$ and *Usp8*$^{f/f}$*Cd4*-Cre thymocytes stimulated with anti-CD3 plus anti-CD28. Numbers adjacent to outlined areas (bottom right) indicate percent TCRβ$^+$Ki67$^+$ cells (top) or TCRβ$^-$Ki67$^+$ cells (bottom). (**b**) IL-2 in supernatants of *Usp8*$^{f/f}$ and *Usp8*$^{f/f}$*Cd4*-Cre thymocytes left unstimulated or stimulated for 72 h with plate-bound anti-CD3. Each symbol represents an individual mouse; small horizontal lines indicate the mean (±s.e.m.). (**c**) Flow cytometry analyzing the expression of CD4 and CD8 on HY-TCR$^+$ thymocytes derived from 7- to 8-week-old female (top) or male (bottom) *Usp8*$^{f/f}$ HY-TCR$^+$ and *Usp8*$^{f/f}$*Cd4*-Cre HY-TCR$^+$ mice. *$P < 0.05$ (paired, two-sided *t*-test (**a**) or unpaired, one-sided *t*-test (**b**)). Data are representative of three independent experiments (error bars (**a**, top right), s.d.).

plasmacytoid dendritic cells (DCs) or conventional DCs in the spleen of *Usp8*$^{f/f}$*Cd4*-Cre mice relative to that of *Usp8*$^{f/f}$ mice (**Fig. 5b**). In contrast, the frequency of T_{reg} cells and plasmacytoid DCs was significantly greater in *Usp8*$^{f/f}$*Cd4*-Cre MLNs than in *Usp8*$^{f/f}$ MLNs (**Fig. 5c**). However, due to the decrease in overall number of T cells, the total number of T_{reg} cells was lower in *Usp8*$^{f/f}$*Cd4*-Cre mice than in *Usp8*$^{f/f}$ mice (**Fig. 5c**). A greater frequency of *Usp8*$^{f/f}$*Cd4*-Cre CD4$^+$ splenocytes than *Usp8*$^{f/f}$ CD4$^+$ splenocytes produced interferon-γ (IFN-γ) and IL-17 after stimulation with PMA and ionomycin (**Fig. 5d**). Moreover, the frequency of cells expressing the activation markers CD25 and CD69 was greater among *Usp8*$^{f/f}$*Cd4*-Cre peripheral CD4$^+$ T cells than among *Usp8*$^{f/f}$ peripheral CD4$^+$ T cells (**Fig. 5e**). Concordantly, expression of the negative activation marker CD45RB was diminished in *Usp8*$^{f/f}$*Cd4*-Cre splenic CD4$^+$ T cells and expression of TCRβ was reduced in *Usp8*$^{f/f}$*Cd4*-Cre CD4$^+$ splenocytes and MLNs relative to that in their *Usp8*$^{f/f}$ counterparts (**Fig. 5e**). These results showed that USP8 was critical for T cell homeostasis *in vivo*.

Colitis in *Usp8*$^{f/f}$*Cd4*-Cre mice

Of note, *Usp8*$^{f/f}$*Cd4*-Cre mice spontaneously developed colitis (**Fig. 6a–c** and **Supplementary Movies 1** and **2**). Pathological and endoscopic analysis revealed colon thickening and inflammation emanating from the distal part of the colon in *Usp8*$^{f/f}$*Cd4*-Cre mice, with enhanced granularity, loss of the vascular pattern of the mucosa and diminished translucency in *Usp8*$^{f/f}$*Cd4*-Cre mice relative to that in *Usp8*$^{f/f}$ mice (**Fig. 6a** and **Supplementary Movies 1** and **2**). We frequently observed intestinal prolapses (data not shown). The colitis was typically accompanied by lymphadenopathy and splenomegaly (**Fig. 6a**). Starting from week 6, mice became moribund and died within 20 weeks, with almost 100% penetrance on the C57BL/6-129/Ola mixed background (**Fig. 6b**). Histological analysis of colon sections stained with hematoxylin and eosin showed thickening of the mucosa, cryptic abscesses, cellular infiltrates, epithelial hyperplasia and elongated crypts in the colon of *Usp8*$^{f/f}$*Cd4*-Cre mice (**Fig. 6c**). Notably, among infiltrating cells, a large portion were CD3$^+$ T cells (**Fig. 6c**). We also detected Foxp3$^+$ T_{reg} cells (**Fig. 6c**). As shown by immunofluorescence, infiltrates of DCs, neutrophils and macrophages were present in inflamed colon sections of *Usp8*$^{f/f}$*Cd4*-Cre mice (**Fig. 6c**). Splenomegaly was characterized by smaller periarteriolar lymphoid sheaths and concomitant enlargement of the Ki67$^+$ red pulp compartment containing Mac-3$^+$ myeloid cells (**Fig. 6d**). These results showed that USP8 in T cells was critical for intestinal immunological homeostasis.

Intrinsic regulation of T cell function by USP8

To analyze the function of USP8 in mature T cells, we crossed *Usp8*$^{f/f}$ mice to mice with transgenic expression of tamoxifen-inducible Cre in CD4$^+$ T cells to generate *Usp8*$^{f/f}$*Cd4*-CreER^{T2} mice. To restrict the deletion of *Usp8* to peripheral T cells, we reconstituted B cell– and T cell–deficient *Rag2*$^{-/-}$*Il2rg*$^{-/-}$ mice with splenocytes from *Usp8*$^{+/+}$*Cd4*-CreER^{T2} or *Usp8*$^{f/f}$*Cd4*-CreER^{T2} mice. Tamoxifen-induced

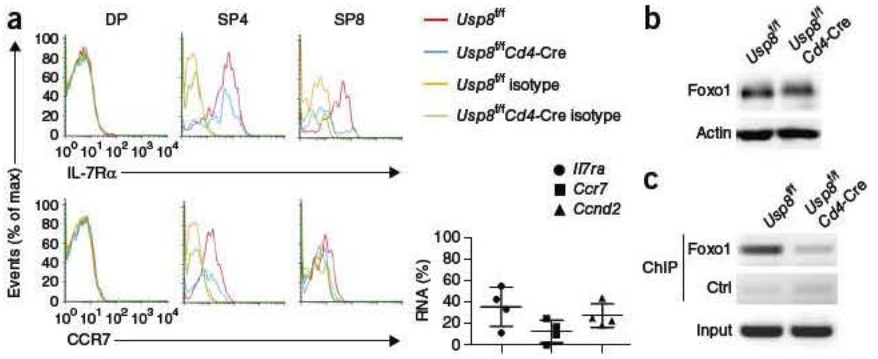

Figure 4 Signaling defects in *Usp8*$^{f/f}$*Cd4*-Cre thymocytes. (**a**) Flow cytometry analyzing the expression of IL-7Rα and CCR7 on *Usp8*$^{f/f}$ and *Usp8*$^{f/f}$*Cd4*-Cre thymocyte subsets (left). Isotype, isotype-matched control antibody. Right (inset), quantitative real-time PCR analysis of *Il7ra* mRNA, *Ccr7* mRNA and *Ccnd2* mRNA (encoding cyclin D2) in *Usp8*$^{f/f}$*Cd4*-Cre DP thymocytes, presented relative to results obtained for *Usp8*$^{f/f}$ DP thymocytes. Each symbol represents an individual mouse; small horizontal lines indicate the mean (±s.d.). $P < 0.001$ (unpaired, two-sided *t*-test). (**b**) Immunoblot analysis of Foxo1 in lysates of *Usp8*$^{f/f}$ and *Usp8*$^{f/f}$*Cd4*-Cre thymocytes purified with anti-CD4. (**c**) Binding of Foxo1 to the *Il7ra* locus in *Usp8*$^{f/f}$ and *Usp8*$^{f/f}$*Cd4*-Cre thymocytes purified with anti-CD4, analyzed by chromatin immunoprecipitation with anti-Foxo1 or a control antibody (Ctrl), followed by PCR with primers for the *Il7ra* enhancer. Input, PCR analysis without immunoprecipitation. Data are representative of at least three independent experiments (**a**, left) or four independent experiments (**a**, inset) or three independent experiments (**b**,**c**).

deletion of *Usp8* resulted in a tenfold reduction in CD4$^+$ splenocytes and CD44^{lo}CD62L^{hi} CD4$^+$ T cells (**Fig. 7a**), which demonstrated that USP8 was essential for maintenance of the naive CD4$^+$ T cell pool. To determine whether USP8-deficient T cells had an intrinsic defect in thymocyte development and peripheral homeostasis, or whether the defect(s) could be overcome by the presence of wild-type T cells, we generated mixed–bone marrow (BM) chimeras by reconstituting irradiated CD45.1$^+$ host mice with wild-type (CD45.1$^+$) BM cells plus *Usp8*$^{f/f}$ (CD45.2$^+$) BM cells or with wild-type (CD45.1$^+$) BM cells plus *Usp8*$^{f/f}$*Cd4*-Cre (CD45.2$^+$) BM cells. Even in the

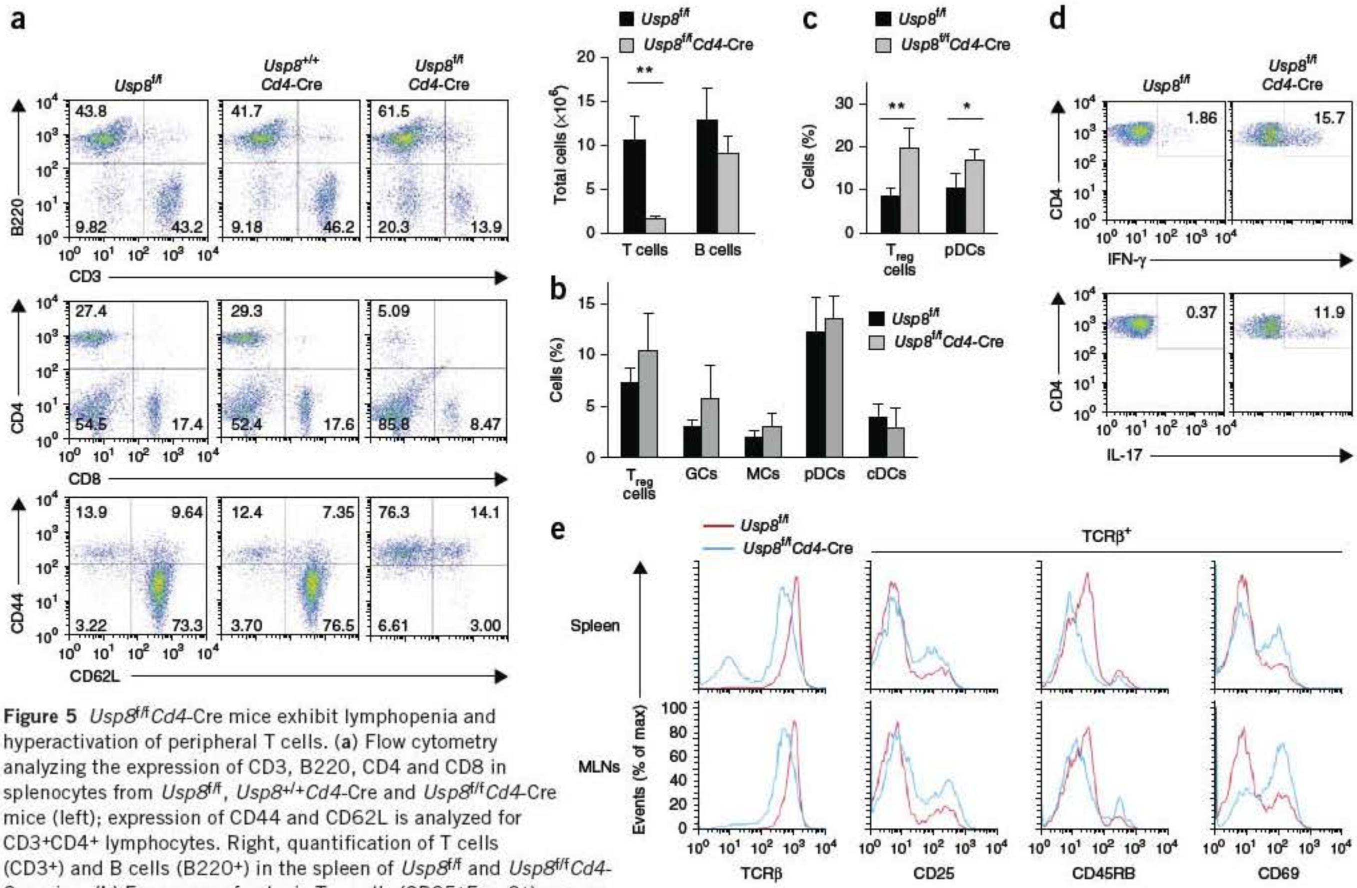

Figure 5 *Usp8*$^{f/f}$*Cd4*-Cre mice exhibit lymphopenia and hyperactivation of peripheral T cells. (**a**) Flow cytometry analyzing the expression of CD3, B220, CD4 and CD8 in splenocytes from *Usp8*$^{f/f}$, *Usp8*$^{+/+}$*Cd4*-Cre and *Usp8*$^{f/f}$*Cd4*-Cre mice (left); expression of CD44 and CD62L is analyzed for CD3$^+$CD4$^+$ lymphocytes. Right, quantification of T cells (CD3$^+$) and B cells (B220$^+$) in the spleen of *Usp8*$^{f/f}$ and *Usp8*$^{f/f}$*Cd4*-Cre mice. (**b**) Frequency of splenic T_{reg} cells (CD25$^+$Foxp3$^+$) among CD4$^+$cells; granulocytes (GCs) (Ly6G$^+$CD11b$^+$) and monocytes (MCs) (CD115$^+$CD11b$^+$) among CD3$^-$ cells; plasmacytoid DCs (pDCs) (CD11c^{lo}B220$^+$) among CD3$^-$Ly6C$^+$CD11b$^-$ cells; and conventional DCs (cDCs) (CD11c^{hi}Ly6C$^-$) among CD3$^-$MHCII$^+$ cells. (**c**) Frequency of T_{reg} cells and plasmacytoid DCs (as in **b**) in MLNs, analyzed by flow cytometry. (**d**) Expression of IFN-γ and IL-17 by splenic CD4$^+$ T cells stimulated with PMA plus ionomycin, assessed by intracellular staining and flow cytometry. Numbers in outlined areas indicate percent IFN-γ$^+$ cells (top) or IL-17$^+$ cells (bottom). (**e**) Flow cytometry analyzing expression of the markers TCRβ, CD25, CD45RB and CD69 on CD4$^+$ lymphocytes (far left) and CD4$^+$ TCRβ$^+$ lymphocytes (middle and right) derived from the spleen and MLNs of *Usp8*$^{f/f}$ and *Usp8*$^{f/f}$*Cd4*-Cre mice. $^*P < 0.01$ and $^{**}P < 0.001$ (unpaired, two-sided *t*-test). Data are representative of six independent experiments (**a**–**c**; mean and s.d.) or at least three (**d**) or six (**e**) independent experiments.

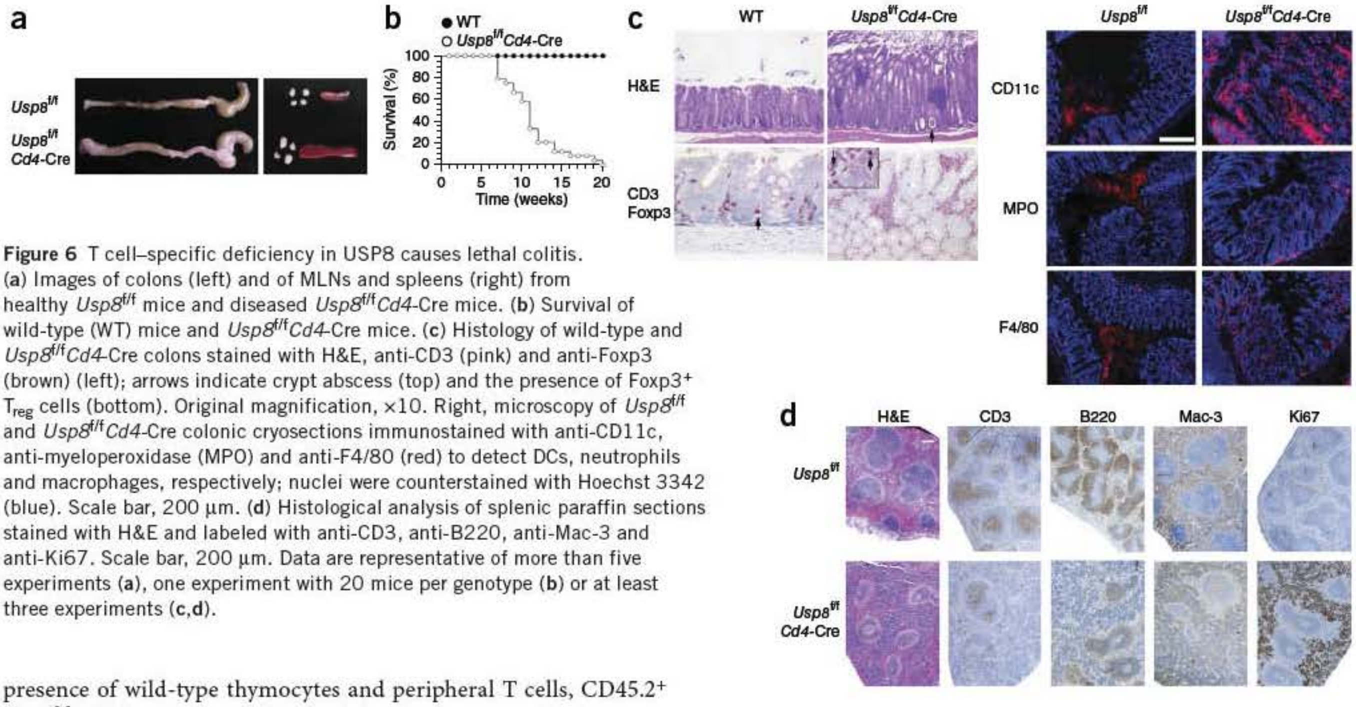

Figure 6 T cell–specific deficiency in USP8 causes lethal colitis. (**a**) Images of colons (left) and of MLNs and spleens (right) from healthy *Usp8*$^{f/f}$ mice and diseased *Usp8*$^{f/f}$*Cd4*-Cre mice. (**b**) Survival of wild-type (WT) mice and *Usp8*$^{f/f}$*Cd4*-Cre mice. (**c**) Histology of wild-type and *Usp8*$^{f/f}$*Cd4*-Cre colons stained with H&E, anti-CD3 (pink) and anti-Foxp3 (brown) (left); arrows indicate crypt abscess (top) and the presence of Foxp3$^+$ T_{reg} cells (bottom). Original magnification, ×10. Right, microscopy of *Usp8*$^{f/f}$ and *Usp8*$^{f/f}$*Cd4*-Cre colonic cryosections immunostained with anti-CD11c, anti-myeloperoxidase (MPO) and anti-F4/80 (red) to detect DCs, neutrophils and macrophages, respectively; nuclei were counterstained with Hoechst 3342 (blue). Scale bar, 200 µm. (**d**) Histological analysis of splenic paraffin sections stained with H&E and labeled with anti-CD3, anti-B220, anti-Mac-3 and anti-Ki67. Scale bar, 200 µm. Data are representative of more than five experiments (**a**), one experiment with 20 mice per genotype (**b**) or at least three experiments (**c**,**d**).

presence of wild-type thymocytes and peripheral T cells, CD45.2$^+$ *Usp8*$^{f/f}$*Cd4*-Cre T cells exhibited a defect in thymocyte development, population expansion and survival in the periphery, accompanied by a shift toward effector and memory populations (**Fig. 7b**). This shift was less severe than that in non-chimeric *Usp8*$^{f/f}$*Cd4*-Cre mice (**Fig. 5a**), which indicated that the effect partially depended on lymphopenia-induced homeostatic expansion. More peripheral *Usp8*$^{f/f}$*Cd4*-Cre (CD45.2$^+$) T cells than wild-type (CD45.1$^+$) T cells produced IFN-γ and IL-17 upon treatment with PMA plus ionomycin (**Fig. 7b**). The surface expression of IL-7Rα was impaired on *Usp8*$^{f/f}$*Cd4*-Cre (CD45.2$^+$) SP thymocytes relative to that on wild-type (CD45.1$^+$) cells (data not shown), which suggested that depletion of USP8 directly affected IL-7Rα expression. IL-7Rα expression was not impaired in peripheral CD45.2$^+$ *Usp8*$^{f/f}$*Cd4*-Cre naive T cells of the competitive BM chimeras described above relative to that in their wild-type (CD45.1$^+$) counterparts (data not shown), which suggested that the reduced IL-7Rα expression on naive T cells, as seen on the non-competitive background, was influenced by extrinsic mechanisms. Together these findings identified USP8 as an intrinsic regulator of both thymocyte development and peripheral T cell homeostasis.

USP8 is dispensable for canonical TCR signaling

To analyze signaling in T cells upon deletion of *Usp8* before the manifestation of secondary effects on cell proliferation and CD3 expression, we took advantage of the 4-hydroxytamoxifen (OHT)-inducible CreER^{T2} system. We observed maximum depletion of USP8 protein in cultured *Usp8*$^{f/f}$*Cd4*-CreER^{T2} CD4$^+$ T cells as early as 48 h after the addition of OHT, relative to its expression in *Usp8*$^{f/+}$*Cd4*-CreER^{T2} control cells (**Fig. 7c**, top left), whereas a reduction in the frequency of CD4$^+$, CD3$^+$ and Ki67$^+$ cells became apparent starting at 72 h after the addition of OHT (**Fig. 7c**). Therefore, at 48 h after OHT treatment, we restimulated CD4$^+$ T cells of each genotype with anti-CD3 plus anti-CD28 and monitored the phosphorylation, activation state and expression of signaling molecules. The phosphorylation of Lat and PLC-γ1 was readily detectable in unstimulated cells of each genotype and was downregulated upon restimulation, which correlated with activation of the kinases Erk1 and Erk2 (**Supplementary Fig. 7a**). Moreover, the expression of Gads and 14-3-3β, phosphorylation of IκB and the kinases p38 and PKB, global Lys48 (K48)-linked ubiquitination, tyrosine-phosphorylation and phosphorylation of SLP-76 (at Ser376) and Jnk, and expression of Foxo1 and GRAIL protein were similar in cells of each genotype (**Supplementary Fig. 7b–d**). Formation of the canonical TCR signalosome was not compromised upon depletion of USP8 (**Supplementary Fig. 8a**).

In mutant cell lines derived from Jurkat human T lymphocytes, lack of SLP-76 did not influence the accumulation of USP8 together with the TCR-CD28 cluster, whereas Lat was crucial for formation of the signalosome (**Supplementary Fig. 8b**). However, the association of USP8 with the TCR-CD28 signaling complex was not abolished in the absence of Gads, which suggested that other interaction partners or motifs of USP8 contributed to binding to the signalosome. Thus, USP8 was dispensable for canonical receptor-proximal signaling induced by CD3-CD28 and, similar to such interactions in mouse T cells, it interacted with the TCR-CD28 cluster in human T cells.

Role of DUB activity and SH3 motifs in USP8 function

To gain insight into the structure-function relationships of USP8 in T cells, we used an *in vivo* 'rescue' approach. For this, we transduced *Usp8*$^{f/f}$*Cd4*-Cre BM stem cells with retrovirus ectopically expressing enhanced green fluorescent protein in combination with wild-type USP8 or USP8 variants lacking catalytic activity (C748A), SH3-binding ability (P405A,P700A) or binding to 14-3-3 (S680A). At 8 weeks after transplantation of those cells into lethally irradiated recipient mice deficient in the RAG-1 recombinase, we analyzed the distribution of splenic T cell subsets. Reconstitution with either empty vector or USP8(C748A) completely failed to 'rescue' T cell development (**Fig. 7d**). Likewise, USP8(P405A,P700A), the variant unable to interact with Gads, showed a significantly reduced ability to 'rescue' T cell development relative to wild-type USP8, which completely restored the T cell compartment (**Fig. 7d**). USP8(S680A) did not show significant defects in the ability to 'rescue' T cell development (**Fig. 7d**). These results showed that both the catalytic activity of USP8 and the

SH3BMs were essential for USP8's function in T cells, but binding of 14-3-3 was not.

USP8 in T_{reg} cell–mediated suppression of γδT cells

The intraepithelial lymphocyte (IEL) population of the gut represents the environmental interface with the greatest abundance of mostly CD8+ γδTCR+ T cells[28]. IEL γδTCR+ T cells are responsible for intestinal inflammation in mice lacking the kinase PDK1 in T cells[24]. PDK1 is also crucial for the maintenance of functional T_{reg} cells. Because our histological analysis revealed disproportionally high recruitment of CD3+ T cells to the inflamed colons of lymphopenic *Usp8*^f/f^*Cd4*-Cre mice (**Fig. 6c**), we investigated whether these were

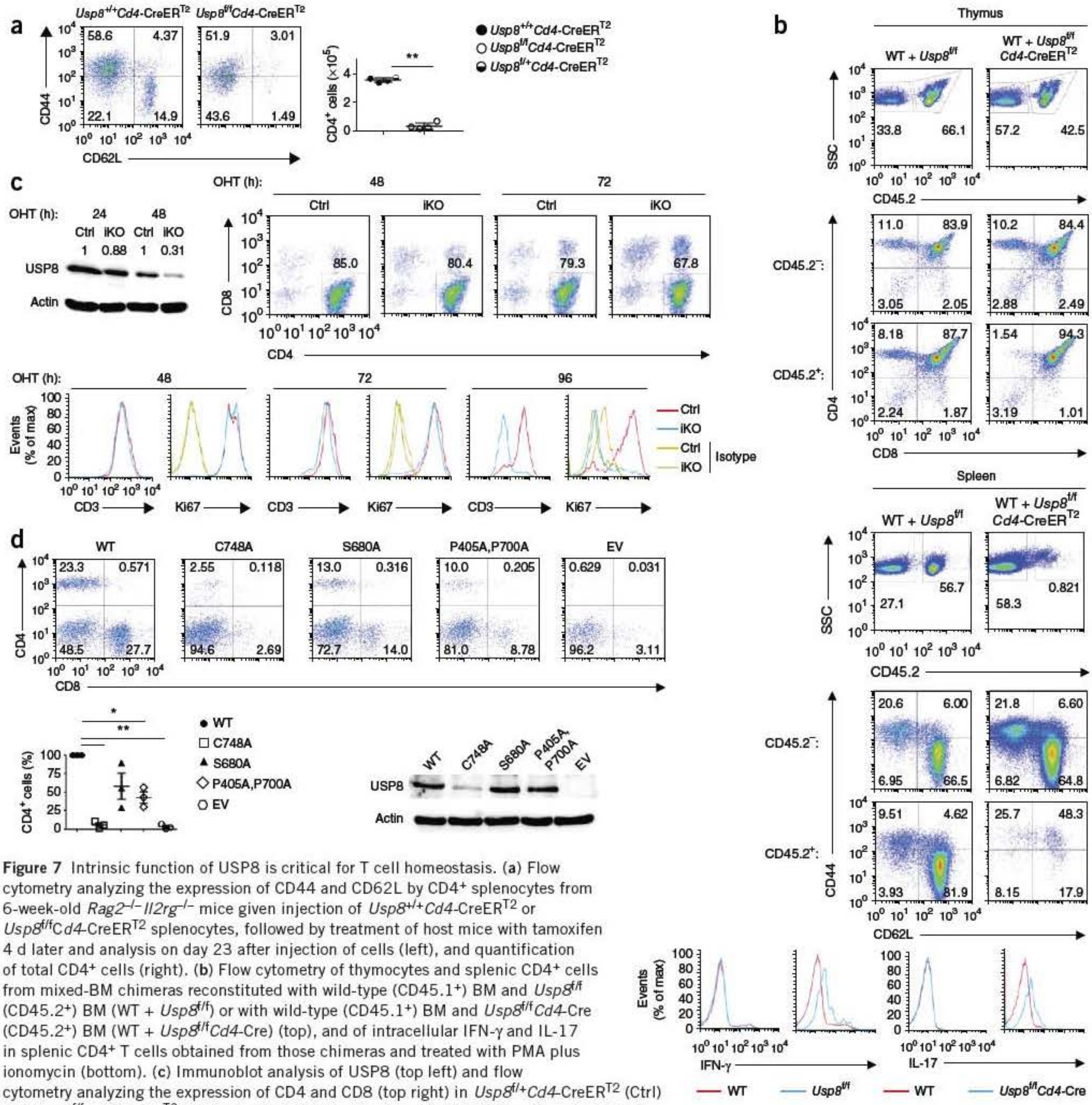

Figure 7 Intrinsic function of USP8 is critical for T cell homeostasis. (**a**) Flow cytometry analyzing the expression of CD44 and CD62L by CD4+ splenocytes from 6-week-old *Rag2*−/− *Il2rg*−/− mice given injection of *Usp8*+/+*Cd4*-CreER^T2^ or *Usp8*f/f*Cd4*-CreER^T2^ splenocytes, followed by treatment of host mice with tamoxifen 4 d later and analysis on day 23 after injection of cells (left), and quantification of total CD4+ cells (right). (**b**) Flow cytometry of thymocytes and splenic CD4+ cells from mixed-BM chimeras reconstituted with wild-type (CD45.1+) BM and *Usp8*f/f (CD45.2+) BM (WT + *Usp8*f/f) or with wild-type (CD45.1+) BM and *Usp8*f/f*Cd4*-Cre (CD45.2+) BM (WT + *Usp8*f/f*Cd4*-Cre) (top), and of intracellular IFN-γ and IL-17 in splenic CD4+ T cells obtained from those chimeras and treated with PMA plus ionomycin (bottom). (**c**) Immunoblot analysis of USP8 (top left) and flow cytometry analyzing the expression of CD4 and CD8 (top right) in *Usp8*f/+*Cd4*-CreER^T2^ (Ctrl) and *Usp8*f/f*Cd4*-CreER^T2^ (iKO) splenic CD4+ T cells stimulated for 48 h with anti-CD3 plus anti-CD28, followed by treatment for various times (above lanes or plots) with OHT in the presence of IL-2; bottom, expression of CD3 and Ki67 in CD4+ cells treated for 48, 72 or 96 h (above plots) with OHT. Isotype, isotype-matched control antibody. (**d**) Flow cytometry of splenocytes from *Rag1*−/− host mice reconstituted with *Usp8*f/f*Cd4*-Cre BM stem cells transduced with retrovirus expressing wild-type or mutant USP8, or empty vector (EV) (above plots), analyzed 8 weeks after reconstitution, as well as frequency of CD4+ lymphocytes among splenocytes in the host mice (bottom left) and immunoblot analysis of retroviral expression of the vectors in NIH3T3 mouse fibroblasts (bottom right). Each symbol (**a**, right; **d**, bottom left) represents an individual mouse; small horizontal lines indicate the mean (±s.d. (**a**) or s.e.m. (**d**)). $*P < 0.01$ and $**P < 0.001$ (unpaired, two-sided *t*-test). Data are representative of four independent experiments (**a**), three independent experiments (**b**,**d**) or at least three independent experiments (**c**).

αβTCR+ cells or γδTCR+ cells. Indeed, CD8+ γδTCR+ T cells were the main T cell population among *Usp8*$^{f/f}$*Cd4*-Cre IELs in inflamed colons (**Fig. 8a**). In contrast to its compromised expression in *Usp8*$^{f/f}$*Cd4*-Cre αβTCR+ T cells, surface expression of the activation marker CD69 was not compromised in γδTCR+ T cells (**Fig. 8a**). We also found an elevated frequency of CD8+ γδTCR+ T cells in the MLNs of *Usp8*$^{f/f}$*Cd4*-Cre mice relative to that in *Usp8*$^{f/f}$ MLNs (**Fig. 8b**). However, more *Usp8*$^{f/f}$*Cd4*-Cre αβTCR+ T cells than *Usp8*$^{f/f}$ αβTCR+ T cells expressed CD69 in MLNs (**Fig. 8b**). The number of γδ T cells was eight- to tenfold higher in the MLNs of both *Usp8*$^{f/f}$*Cd4*-Cre mice (**Fig. 8c**) and *Usp8*$^{f/f}$*Lck*-Cre mice (data not shown) than in *Usp8*$^{f/f}$ MLNs, and this number was fourfold

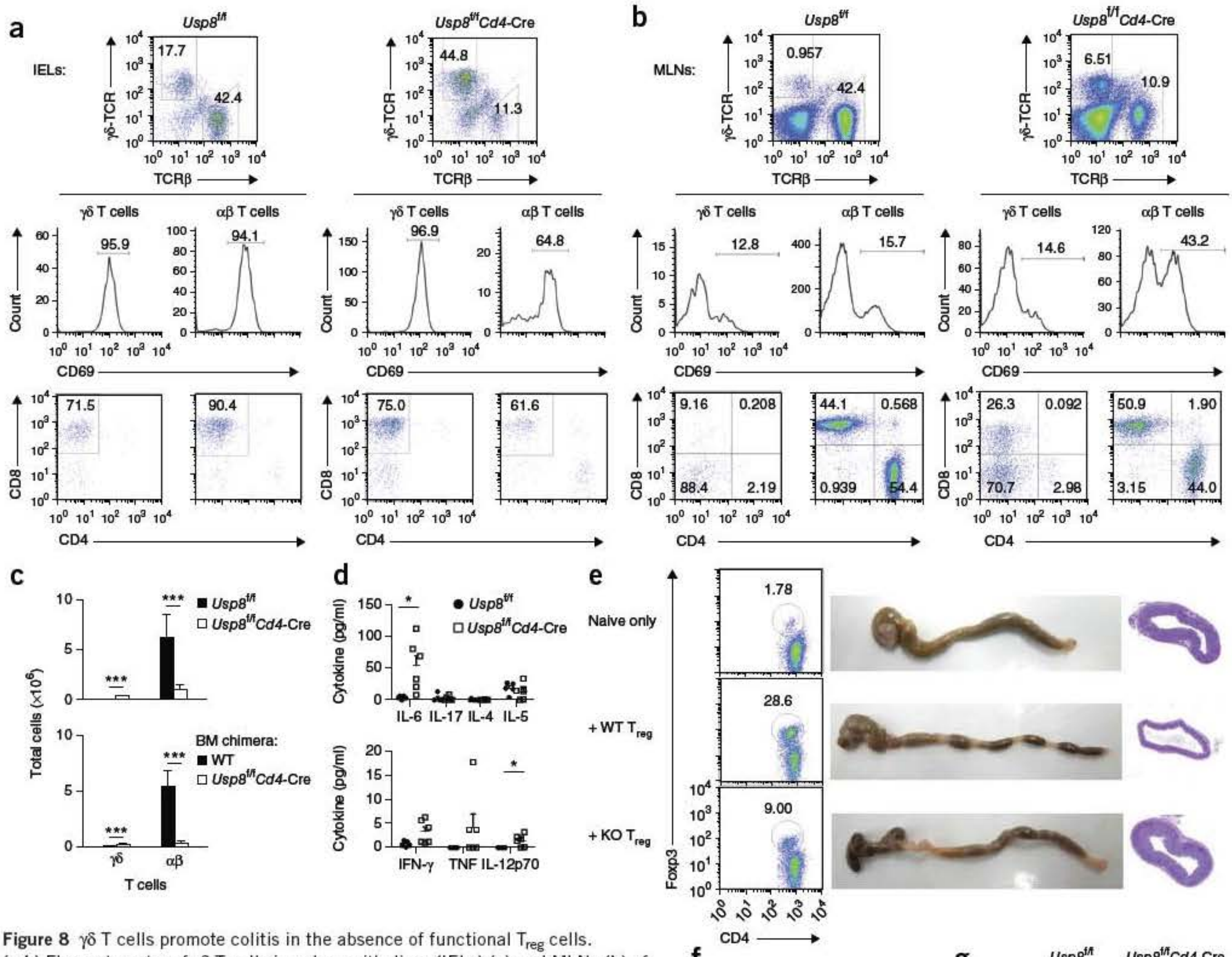

Figure 8 γδ T cells promote colitis in the absence of functional T_{reg} cells. (**a**,**b**) Flow cytometry of γδ T cells in colon epithelium (IELs) (**a**) and MLNs (**b**) of *Usp8*$^{f/f}$ mice and diseased *Usp8*$^{f/f}$Cd4-Cre mice. Numbers adjacent to outlined areas (top row) indicate percent γδTCR+TCRβ− cells (top left) or γδTCR−TCRβ+ cells (bottom right) among total IELs (**a**) or total MLN cells (**b**); numbers above bracketed lines (middle row) indicate percent CD69+ cells in various subsets (above plots) of IELs (**a**) or MLN cells (**b**); numbers adjacent to outlined areas (bottom row) indicate percent CD8+CD4− (SP8) cells in those subsets of IELs (**a**) or MLN cells (**b**). (**c**) Total αβ and γδ T cells in MLNs of 12-week-old *Usp8*$^{f/f}$ mice ($n = 5$) or *Usp8*$^{f/f}$*Cd4*-Cre mice ($n = 6$), or chimeras reconstituted with wild-type (CD45.1+) BM plus *Usp8*$^{f/f}$*Cd4*-Cre (CD45.2+) BM ($n = 6$; results normalized to 100% reconstitution efficiency for each genotype). (**d**) Cytokine concentrations in serum from 3- to 4-month-old healthy *Usp8*$^{f/f}$ mice and *Usp8*$^{f/f}$*Cd4*-Cre mice with colitis. (**e**) Foxp3 expression in splenic CD4+ cells from *Rag1*$^{-/-}$ host mice 9 weeks after injection of *Usp8*$^{f/f}$ naive T cells alone (Naive only) or together with *Usp8*$^{f/f}$ T_{reg} cells (+ WT T_{reg}) or *Usp8*$^{f/f}$*Cd4*-Cre T_{reg} cells (+ KO T_{reg}) (left), and macroscopic (middle) and microscopic (right) analysis of colons with staining of distal sections by H&E. (**f**) Division of responding T cells (T_{resp}) stimulated with anti-CD3 and cultured alone (T_{resp} only) or in the presence of *Usp8*$^{f/f}$ or *Usp8*$^{f/f}$*Cd4*-Cre T_{reg} cells, assessed as dilution of the division-tracking dye CFSE. (**g**) IFN-γ expression in CD4+Foxp3+ splenocytes obtained from 7-week-old *Usp8*$^{f/f}$ and *Usp8*$^{f/f}$*Cd4*-Cre mice ($n = 5$ per genotype) and-stimulated with PMA plus ionomycin. $^{*}P < 0.05$, $^{**}P < 0.01$ and $^{***}P < 0.001$ (unpaired (**c**,**d**) or paired (**g**) two-sided *t*-test). Data are representative of two independent experiments (**a**,**b**), at least five experiments (**c**,**d**; error bars, s.d. (**c**) or s.e.m. (**d**)), at least three independent experiments (**e**), two independent experiments each with technical triplicates of cells from four (*Usp8*$^{f/f}$) or ten (*Usp8*$^{f/f}$*Cd4*-Cre) mice (**f**) or five independent experiments (**g**; error bars, s.d.).

higher in the *Usp8*$^{f/f}$*Cd4*-Cre T cell population of mixed BM chimeras than in their wild-type (CD45.1$^+$) counterparts (**Fig. 8c**). Furthermore, quantification of cytokines in blood revealed that concentrations of the pro-inflammatory cytokines IL-6 and IL-12p70 were significantly higher in the serum of diseased *Usp8*$^{f/f}$*Cd4*-Cre mice than in that of healthy *Usp8*$^{f/f}$ mice (**Fig. 8d**). We also detected elevated concentrations of IFN-γ and tumor-necrosis factor in *Usp8*$^{f/f}$*Cd4*-Cre mice relative to their concentrations in *Usp8*$^{f/f}$ mice, whereas we did not observe enhanced expression of IL-10 or IL-17 (**Fig. 8d** and data not shown).

To determine whether USP8 is crucial for the function of T_{reg} cells, we performed an *in vivo* suppression assay. The co-injection of *Usp8*$^{f/f}$*Cd4*-Cre T_{reg} cells did not prevent the onset of colitis in *Rag1*$^{-/-}$ mice reconstituted with naive CD4$^+$ *Usp8*$^{f/f}$ cells (**Fig. 8e**). USP8-deficient T_{reg} cells were also less effective than USP8-sufficient T_{reg} cells in inhibiting the proliferation of naive T cells *in vitro* (**Fig. 8f**). T_{reg} cell–specific deficiency in Foxo1 results in dysfunctional cells due to deregulated IFN-γ responses[20]. Likewise, we observed an enhanced IFN-γ response in T_{reg} cells depleted of USP8 relative to the response of USP8-sufficient T_{reg} cells (**Fig. 8g**). These data demonstrated that USP8 was required for the maintenance of enteric immunotolerance by T_{reg} cells via suppression of an inflammatory response mediated by intestinal $\gamma\delta$ T cells.

DISCUSSION

Here we have identified USP8 as a previously unknown component of the TCR signaling complex. Gads binds with higher affinity to an SH3BM of USP8 than does STAM2 and has high expression in lymphocytes[9,10]. This might explain why the stability of STAM2 and endosomal sorting seemed to be unaffected in T cells depleted of USP8. Ectopic re-expression of an USP8 mutant lacking the SH3BMs did not restore T cell development. This showed that the SH3BM-mediated interactions were essential for the function of USP8 in T cells and suggested that USP8-Gads binding is critical for the specificity of USP8 function in T cells[5,9,10]. Our *in vivo* rescue experiments also demonstrated that the catalytic activity of USP8 was essential for its function in T cell development. This is of particular interest, as different DUBs, such as A20 or USP18, have been shown to have physiologically relevant non-enzymatic functions[29–31].

The binding of USP8 to 14-3-3β was dependent on a typical serine-phosphorylation motif within the 14-3-3BM of USP8 (ref. 7). The binding of 14-3-3ε, 14-3-3λ and 14-3-3ζ has been shown to inhibit USP8's catalytic activity[6,7]. Thus, phosphorylation-controlled binding of 14-3-3β might negatively regulate USP8's enzymatic function in T cells. Upon stimulation via the TCR, USP8 is cleaved by a caspase-dependent mechanism. Consistent with the resulting amino-terminal 70-kilodalton cleavage product observed in our study, human mutant USP8 is proteolytically cleaved in the 14-3-3BM, which promotes DUB activity[6]. Thus, it is conceivable that the binding of 14-3-3β and proteolytic processing of USP8 contrarily regulate USP8's DUB activity in a TCR-stimulation–dependent manner.

Our findings suggest that Gads and 14-3-3β represent substrates for USP8-mediated deubiquitination. The unaltered stability of Gads and 14-3-3β in the absence of USP8 indicated that USP8-mediated deubiquitination regulated protein complex composition rather than the proteasomal degradation of these proteins. Of note, TCR-induced interactions of SLP-76 and Gads with proteins of the 14-3-3 family causes uncoupling from the adaptor Lat and inhibits TCR signaling[26,32]. USP8 has been suspected of regulating the stability of GRAIL[15]. However, we did not detect any differences in GRAIL's stability in the absence of USP8.

T cell–specific deletion of *Usp8* caused a defect in SP thymocyte maturation. During the development of SP thymocytes, low-affinity interactions of the TCR with self peptide–MHC trigger a signaling cascade that allows positive selection[11]. Despite the lack of an effect of USP8 depletion on negative selection or TCR-induced canonical signaling cascades, USP8 might be involved in the discrimination of low- and high-affinity signals in specific cell populations. Likewise, USP8 might control stabilization and upregulation of the TCR during development. Thus, USP8 might indirectly influence thymocyte maturation and upregulation of IL-7Rα expression. According to the kinetic signaling model of T cell development, signaling from cytokine receptors specifies the lineage fate of MHC class I–selected thymocytes, whereas TCR signaling specifies that of MHC class II–selected thymocytes[33]. Similar to mice with T cell–specific IL-7R deficiency, *Usp8*$^{f/f}$*Cd4*-Cre mice showed a marked reduction in SP8 cells among TCRβ^{hi} thymocytes relative to the abundance of these cells in *Usp8*$^{f/f}$ mice. However, the defect in the maturation of SP4 cells suggested that IL-7R-independent mechanisms were affected as well.

Our data indicated that USP8 was critical for Foxo1's activity in thymocytes. Regulation of the ubiquitination of 14-3-3β by USP8 might directly regulate the binding of Foxo1 to DNA in the nucleus. Foxo1 controls expression of the gene encoding Foxp3 and a subset of T_{reg} cell–associated genes and is critical for suppressing expression of the gene encoding IFN-γ[20,34]. Thus, impaired Foxo1 activity, as detected in USP8-deficient thymocytes, might provide an explanation for the compromised T_{reg} cell function associated with increased IFN-γ production in *Usp8*$^{f/f}$*Cd4*-Cre mice. Accordingly, in a model of BM transfer into sublethally irradiated *Rag1*$^{-/-}$ mice, lack of Foxo1 expression in T cells resulted in colitis presumably due to defective abundance and function of T_{reg} cells[18]. Consistent with the fact that the gut represents the interface with the greatest abundance of $\gamma\delta$ T cells, the colitis in *Usp8*$^{f/f}$*Cd4*-Cre mice was associated with an impaired suppressive effect of T_{reg} cells on the highly abundant CD8$^+$ $\gamma\delta$ T cells in inflamed colons. $\gamma\delta$ T cells have already been shown to have an exacerbating role in chronic colitis in mice expressing mutant TCRα and in *Pdk1*$^{f/f}$*Cd4*-Cre mice (which have T cell–specific deficiency in phosphoinositide-dependent kinase 1) (refs. 24,35). Similar to *Usp8*$^{f/f}$*Cd4*-Cre mice, mice with T cell–specific deletion of the gene encoding Foxo1 have defects in IL-7-dependent homeostatic proliferation accompanied by spontaneous T cell activation and effector T cell differentiation[18,36]. Moreover, the failure of IL-2 secretion and upregulation of CCR7 expression in thymocytes and the lack of upregulation of IL-10 expression in diseased *Usp8*$^{f/f}$*Cd4*-Cre mice might themselves account for defective *in vivo* T_{reg} cell function and/or susceptibility to colitis[37–39]. In conclusion, we have identified USP8 as a Gads-interacting DUB associated with the TCR signalosome. The previously undefined role of USP8 in T cell development, homeostasis of the immune system and control of the expression of genes that are targets of Foxo1 might provide new avenues for the treatment of immunological disorders.

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AUTHOR CONTRIBUTIONS

A.D. designed and performed experiments, analyzed the data and wrote the paper; A.K. designed and performed experiments; S.N. generated the *Usp8*$^{f/f}$ mice and designed and performed experiments; A.B. performed the yeast two-hybrid screen; S.R. and A.W. contributed to or carried out the endoscopic recording and histological analyses; A.Schö provided help with experiments; A.A. and M.G. contributed to the yeast two-hybrid screen and provided reagents; T.K. performed the gene-expression microarray analysis; A.Schl performed the identification of ubiquitination sites by mass spectrometry; D.Y. generated and provided mutant Jurkat cells; T.B. generated *Cd4*-CreER^{T2} mice; W.W.S. provided reagents and contributed to the calcium-influx experiment; M.P. contributed to the histological analyses; and K.-P.K. supervised the project and wrote the paper.

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The authors declare no competing financial interests.

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