Published in final edited form as:

Nat Immunol. 2016 March ; 17(3): 286–296. doi:10.1038/ni.3352.

SHARPIN controls regulatory T cells by negatively modulating the T cell antigen receptor complex

Yoon Park^{1,4}, Hyung-seung Jin^{1,4}, Justine Lopez¹, Jeeho Lee¹, Lujian Liao², Chris Elly¹, and Yun-Cai Liu^{1,3}

¹Division of Cell Biology, La Jolla Institute for Allergy and Immunology, La Jolla, California, USA

²Institute of Biomedical Sciences, School of Life Sciences, East China Normal University, Shanghai, China

³Institute for Immunology, Peking-Tsinghua Center for Life Sciences, Tsinghua University, Beijing, China

Abstract

SHARPIN forms a linear-ubiquitin-chain-assembly complex that promotes signaling via the transcription factor NF- κ B. SHARPIN deficiency leads to progressive multi-organ inflammation and immune system malfunction, but how SHARPIN regulates T cell responses is unclear. Here we found that SHARPIN deficiency resulted in a substantial reduction in the number of and defective function of regulatory T cells (T_{reg} cells). Transfer of SHARPIN-sufficient T_{reg} cells into SHARPIN-deficient mice considerably alleviated their systemic inflammation. SHARPIN-deficient T cells displayed enhanced proximal signaling via the T cell antigen receptor (TCR) without an effect on the activation of NF- κ B. SHARPIN conjugated with Lys63 (K63)-linked ubiquitin chains, which led to inhibition of the association of TCR ζ with the signaling kinase Zap70; this affected the generation of T_{reg} cells. Our study therefore identifies a role for SHARPIN in TCR signaling whereby it maintains immunological homeostasis and tolerance by regulating T_{reg} cells.

Ubiquitination is an important post-translational modification for the regulation of many processes and is catalyzed by a three-step enzymatic cascade that involves E1, E2, and ubiquitin ligase (E3) enzymes¹. Ubiquitin can be conjugated to another ubiquitin through the formation of isopeptide bond between the carboxy-terminal glycine residue of one ubiquitin and a lysine residue (Lys6 (K6), K11, K27, K29, K33, K48 or K63) or amino-terminal

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Correspondence should be addressed to Y.-C.L. (yuncail@lji.org).

⁴These authors contributed equally to this work.

AUTHOR CONTRIBUTIONS

Y. P. and H.J. designed and performed the experiments, analyzed the data, and wrote the manuscript; J. Lo. and C.E. did the mouse breeding and helped with the preparation of experiments; J. Le. helped with experimental design and data analysis; L.L. performed the proteomics analysis; and Y.-C.L. initiated and secured funding for this project, helped with experimental design and data interpretation, and wrote the manuscript.

COMPETING FINANCIAL INTERESTS

The authors declare no competing financial interests.

methionine residue of the preceding ubiquitin (linear ubiquitin), which leads to the assembly of polyubiquitin chain of different linkages with distinct biological functions^{2,3}.

SHARPIN was initially identified in the excitatory synapses in the rat brains⁴; it forms a linear-ubiquitin-chain-assembly complex (LUBAC), together with the LUBAC components HOIP and HOIL-1. The linear ubiquitin chains positively regulate activation of the transcription factor NF- κ B in signaling via tumor-necrosis factor (TNF) and IL-1 β ⁵⁻⁷. Spontaneous null mutation of the mouse gene encoding SHARPIN (*Sharpin*; called '*Cpdm*' here) leads to chronic proliferative dermatitis, multi-organ inflammation, and malfunction of the immune system^{8,9}. However, another study has reported that increased IL-1 β -mediated activation of NF- κ B results in dermatitis in SHARPIN-deficient (*Cpdm*^{-/-}) mice¹⁰. Moreover, HOIL-1-deficient mice do not develop inflammation under homeostatic conditions¹¹. Indeed, LUBAC-independent functions of SHARPIN have been identified in integrin signaling^{12,13}. It has also been shown that with the exception of cutaneous inflammation, the systemic multi-organ inflammation in SHARPIN-deficient mice is driven mainly by B lymphocytes and T lymphocytes¹⁴. Therefore, the exact mechanisms of SHARPIN in controlling immunological homeostasis remain unclear.

Regulatory T cells (T_{reg} cells) serve a central role in maintaining immunotolerance and homeostasis and are characterized by expression of the transcription factor Foxp3, which is critically involved in their development and function¹⁵⁻¹⁷. Impaired generation of T_{reg} cells leads to severe autoimmune and/or inflammatory diseases in both humans and mice¹⁷⁻¹⁹. Signaling via the T cell antigen receptor (TCR) has a pivotal role in Foxp3 expression during the thymic development and peripheral differentiation of T_{reg} cells and in their suppressive activity²⁰. Engagement of the TCR induces the phosphorylation of immunoreceptor tyrosine-based activation motifs (ITAMs) of the TCR ζ chain and recruitment of the signaling kinase Zap70 and the activation of downstream molecules for T cell activation²¹. Mutations in the gene encoding Zap70 and the adaptor LAT result in defective T_{reg} cell development and autoimmune diseases²²⁻²⁵, whereas mutation resulting in signaling-deficient TCR ζ increases the number and suppressive activity of T_{reg} cells²⁶. Such studies suggest the importance of proximal TCR signaling in the regulation of T_{reg} cells.

In this study, we found that *Cpdm*^{-/-} mice had significantly fewer T_{reg} cells than did *Cpdm*^{+/-} mice in both the thymus and peripheral organs. Molecular studies revealed that SHARPIN negatively regulated TCR signaling by inhibiting interactions between TCR ζ and Zap70. Our findings suggest a critical role for SHARPIN in the development and function of T_{reg} cells that contributes to the control of immunological homeostasis and inflammation.

RESULTS

Altered T cell activation in *Cpdm*^{-/-} mice

To understand how SHARPIN is involved in controlling immunological homeostasis, we assessed the effect of SHARPIN deficiency on T cells. 6-week-old *Cpdm*^{-/-} mice displayed substantial infiltration of leukocytes into the lungs and small intestine (Fig. 1a). Given the high expression of SHARPIN in various T cell subpopulations (Supplementary Fig. 1a), we reasoned that T cells might have caused this inflammation. *Cpdm*^{-/-} mice had fewer total

thymocytes than did *Cpdm*^{+/-} mice (Supplementary Fig. 1b). The proportion of CD4⁺ single-positive thymocytes was slightly greater in *Cpdm*^{-/-} mice than in *Cpdm*^{+/-} mice, and a normal amount of apoptosis was observed in *Cpdm*^{-/-} CD4⁺CD8⁺ double-positive thymocytes after stimulation via the TCR and coreceptor CD28 (Supplementary Fig. 1c,d). However, the number of CD4⁺ T cells and proportion of memory T cells was substantially greater in the lungs of *Cpdm*^{-/-} mice than in those of *Cpdm*^{+/-} mice (Fig. 1b,c). After stimulation via the TCR or the TCR and CD28, SHARPIN-deficient T cells displayed greater proliferative capacity than that of wild-type T cells but an amount of apoptosis similar to that of wild-type T cells (Fig. 1d,e). We assessed cytokines characteristic of the TH2 and TH17 subsets of helper T cells and detected elevated production of such cytokines by CD4⁺ T cells in the lungs of *Cpdm*^{-/-} mice, relative to that in the lungs of *Cpdm*^{+/-} mice, while the production of interferon- γ (IFN- γ) by CD4⁺ T cells was lower in the lungs of *Cpdm*^{-/-} mice than in those of *Cpdm*^{+/-} mice (Fig. 1f), consistent with a published report²⁷.

Requirement for SHARPIN in T_{reg} cell generation

We then analyzed Foxp3 expression in T_{reg} cells. Notably, the frequency and number of T_{reg} cells were substantially lower in all organs analyzed in 4-week-old *Cpdm*^{-/-} mice than in their *Cpdm*^{+/-} counterparts (Fig. 2a,b). We then generated chimeric mice by reconstituting *Rag1*^{-/-} (CD45.1⁺) mice (which have a congenital deficiency in mature B cells and T cells) with bone marrow (BM) cells from *Cpdm*^{+/-} (CD45.2⁺) mice or *Cpdm*^{-/-} (CD45.2⁺) mice and generated mixed chimeric mice by reconstituting the *Rag1*^{-/-} (CD45.2⁺) mice instead with a mixture of BM cells from wild-type (CD45.1⁺) mice and *Cpdm*^{-/-} (CD45.2⁺) mice. All chimeric mice generated showed normal T cell development (Supplementary Fig. 2a,b). However, *Cpdm*^{-/-} T_{reg} cells were nearly absent from both the thymus and the spleen, in contrast to the presence of wild-type T_{reg} cells (Fig. 2c). Although the proliferation of *Cpdm*^{-/-} T cells was greater than that of *Cpdm*^{+/-} T cells after stimulation via the TCR or the TCR and CD28, more CD62L⁺ CD4⁺ T cells were present in CD45.2⁺ (*Cpdm*^{-/-}) populations than in CD45.1⁺ (wild-type) populations in the mixed chimeric mice (Supplementary Fig. 2c,e); however, SHARPIN-deficient CD4⁺ T cell populations in chimeric mice reconstituted with *Cpdm*^{-/-} BM showed a greater frequency of memory-phenotype cells and cytokine production than did SHARPIN-sufficient CD4⁺ T cells in chimeric mice reconstituted with *Cpdm*^{+/-} BM, accompanied by lung inflammation in the chimeric mice reconstituted with *Cpdm*^{-/-} BM (Supplementary Fig. 2f,g).

We then assessed the effect of SHARPIN deficiency on the differentiation of CD4⁺ T cells to T_{reg} cells *in vitro*. Although the responsiveness of *Cpdm*^{-/-} CD4⁺ T cells to signaling via transforming growth factor- β (TGF- β) was normal (Fig. 2d and Supplementary Fig. 2h), Foxp3 expression was not efficiently induced in *Cpdm*^{-/-} CD4⁺ T cells after stimulation with either a low dose or a high dose of antibody to the invariant signaling protein CD3 (anti-CD3) (Fig. 2e). We further assessed the ability of SHARPIN to generate antigen-induced T_{reg} cells *in vivo* by performing adoptive-transfer experiments²⁸. The frequency of antigen-induced T_{reg} cells was significantly lower in mice that received *Cpdm*^{-/-} CD4⁺ T

Note: Any Supplementary Information and Source Data files are available in the [online version of the paper](#).

cells than in those that received *Cpdm*^{+/-} CD4⁺ T cells (Fig. 2f). Collectively, these data indicated an intrinsic role for SHARPIN in the generation of T_{reg} cells.

Requirement for SHARPIN for T_{reg} cell function and stability

We next investigated whether the decreased number of T_{reg} cells in *Cpdm*^{-/-} mice was caused by altered proliferation or apoptosis. In the periphery, the frequency of cells expressing the proliferation marker Ki67 and the rate of *in vitro* cell division after stimulation via the TCR were almost completely equivalent in *Cpdm*^{+/-} T_{reg} cells and *Cpdm*^{-/-} T_{reg} cells (Supplementary Fig. 3a,b). The rate of apoptosis was similar in *Cpdm*^{+/-} CD4⁺CD25⁺ Nrp1⁺ splenic T_{reg} cells and their *Cpdm*^{-/-} counterparts under steady-state conditions or after stimulation via the TCR (Supplementary Fig. 3c). Similar results were observed for the mixed chimeric mice reconstituted with BM cells from wild-type mice and *Cpdm*^{-/-} mice (Supplementary Fig. 3d,e). However, the apoptosis of SHARPIN-deficient thymic T_{reg} cells in *Cpdm*^{-/-} mice was greater than that of SHARPIN-sufficient thymic T_{reg} cells in *Cpdm*^{+/-} mice under steady-state conditions (Supplementary Fig. 3f) or in mixed chimeric mice reconstituted with BM cells from wild-type mice and *Cpdm*^{-/-} mice (Supplementary Fig. 3g).

We then analyzed the expression patterns of surface markers on T_{reg} cells. The surface markers CD25, CD44, CD103, CTLA4, ICOS and GITR displayed higher expression on *Cpdm*^{-/-} T_{reg} cells than on *Cpdm*^{+/-} T_{reg} cells (Fig. 3a), probably due to the continuing inflammation in *Cpdm*^{-/-} mice. We next crossed *Cpdm*^{-/-} or *Cpdm*^{+/-} mice with *Foxp3*^{YFP^{Cre}} mice, to mark T_{reg} cells with a yellow fluorescent protein (YFP) reporter, then obtained cells from the *Foxp3*^{YFP^{Cre}} *Cpdm*^{-/-} or *Foxp3*^{YFP^{Cre}} *Cpdm*^{+/-} progeny and performed *in vitro* co-culture suppression assays (Fig. 3b). *Cpdm*^{-/-} T_{reg} cells and *Cpdm*^{+/-} T_{reg} cells suppressed the division of naive CD4⁺ T cells to a similar extent (Fig. 3c). To further investigate the function of *Cpdm*^{-/-} T_{reg} cells *in vivo*, we induced colitis by adoptive transfer of sorted CD4⁺CD45RB^{hi} (CD45.1⁺) naive T cells together with YFP⁺CD25⁺ (CD45.2⁺) T_{reg} cells from *Foxp3*^{YFP^{Cre}} *Cpdm*^{+/-} or *Foxp3*^{YFP^{Cre}} *Cpdm*^{-/-} mice into *Rag1*^{-/-} mice. In contrast to *Cpdm*^{+/-} T_{reg} cells, *Cpdm*^{-/-} T_{reg} cells failed to suppress colitis, as assessed by loss of body weight, colon histology and colon length shortening (Fig. 3d,e and Supplementary Fig. 4a). In addition, we observed a higher ratio of CD4⁺ T cells to T_{reg} cells among *Cpdm*^{-/-} cells than among *Cpdm*^{+/-} cells (Supplementary Fig. 4b), but without diminished proliferation or migratory ability of *Cpdm*^{-/-} T_{reg} cells relative to that of *Cpdm*^{+/-} T_{reg} cells (Supplementary Fig. 4c,e,f), as suggested before¹². Greater production of IFN-γ and IL-17 by CD45.1⁺CD4⁺ T cells was also detected in mice that received *Cpdm*^{-/-} T_{reg} cells than in those that received *Cpdm*^{+/-} T_{reg} cells in the adoptive-transfer model of colitis (Supplementary Fig. 4d). Furthermore, in this colitis model, *Cpdm*^{-/-} T_{reg} cells exhibited a substantial loss of Foxp3 expression relative to its expression by *Cpdm*^{+/-} T_{reg} cells (Fig. 3f). Although sorted Foxp3⁺ T_{reg} cells from *Cpdm*^{+/-} mice and *Cpdm*^{-/-} mice exhibited largely similar demethylation of the conserved noncoding sequence CNS2 of *Foxp3* (Supplementary Fig. 5a), spleen and lung *Cpdm*^{-/-} Foxp3⁺ T_{reg} cells showed a markedly more IL-17 production than that of their *Cpdm*^{+/-} counterparts (Fig. 3g,h). More IL-17 production was also observed in *Cpdm*^{-/-} T_{reg} cells than in *Cpdm*^{+/-} T_{reg} cells in mixed chimeric mice reconstituted with BM cells from *Cpdm*^{+/-} mice and *Cpdm*^{-/-} mice

(Supplementary Fig. 5b). We also observed higher expression of the transcription factor ROR γ t in *Cpdm*^{-/-} CD4⁺ T cells than in *Cpdm*^{+/-} CD4⁺ T cells under T_{reg} cell-polarizing conditions (Supplementary Fig. 5c). However, IL-17 production was similar in *Cpdm*^{+/-} conventional CD4⁺ non-T_{reg} cells and their *Cpdm*^{-/-} counterparts (Supplementary Fig. 5d,f). These results suggested that SHARPIN was required for maintaining the suppressive activity of T_{reg} cells.

T_{reg} cell-mediated suppression of inflammation in *Cpdm*^{-/-} mice

The impaired generation of T_{reg} cells leads to severe autoimmune and inflammatory disease in both mice and humans^{15,29}. To determine whether the reduced number and impaired function of T_{reg} cells drove the inflammatory phenotype of *Cpdm*^{-/-} mice, we sorted CD4⁺CD25⁺YFP⁺ T_{reg} cells from *Foxp3*^{YFP}Cre *Cpdm*^{+/+} mice by flow cytometry, adoptively transferred the sorted cells into a cohort of 1-day old (neonatal) *Cpdm*^{-/-} mice by intraperitoneal injection and analyzed them 6 weeks after transfer. *Cpdm*^{-/-} mice that received those T_{reg} cells exhibited alleviated inflammation (Fig. 4a) and a lower total number of inflammatory cells in the lungs compared with that of *Cpdm*^{-/-} mice that received no cells (Fig. 4b). Moreover, the proportion of CD62L⁺ and CD44⁺ lung CD4⁺ T cells in *Cpdm*^{-/-} mice that received wild-type T_{reg} cells was restored to the normal frequency found in *Cpdm*^{+/-} mice that received no cells (Fig. 4c). Notably, T_H2 and T_H17-type cytokine production was also reduced by injection of exogenous wild-type T_{reg} cells into *Cpdm*^{-/-} mice, compared with the production of these cytokines in *Cpdm*^{-/-} mice that received no cells (Fig. 4d). These data suggested an intrinsic role for *Cpdm*^{-/-} T_{reg} cells in the development of inflammation.

Negative regulation of TCR signaling by SHARPIN

Published studies have reported that the activation of NF- κ B is abrogated in *Cpdm*^{-/-} cells after stimulation with TNF and IL-1 β ⁵⁻⁷. However, the rate at which the inhibitory cytoplasmic NF- κ B chaperone I κ B α was degraded and the amount of phosphorylation of I κ B α was similar in SHARPIN-deficient T cells and SHARPIN-sufficient T cells after stimulation via the TCR and CD28, the phorbol ester PMA plus ionomycin, or TNF (Fig. 5a). Analysis of phosphorylation of the NF- κ B subunit p65 by flow cytometry also revealed unaltered activation of NF- κ B in *Cpdm*^{-/-} T cells after stimulation with various stimuli, compared with its activation in their *Cpdm*^{+/-} counterparts (Fig. 5b). Notably, after stimulation via the TCR or the TCR and CD28, phosphorylation of TCR ζ , as well as that of its downstream molecules (including Zap70, LAT, ERK1/2, JNK1/2, Akt and Foxo1, but not Lck or PLC γ 1), was much greater in *Cpdm*^{-/-} CD4⁺ T cells, as well as in *Cpdm*^{-/-} T_{reg} cells and *Cpdm*^{-/-} CD4⁺CD8⁺ double-positive thymocytes, than in their *Cpdm*^{+/-} counterparts (Fig. 5c,d and Supplementary Fig. 6b,c). However, stimulation of *Cpdm*^{-/-} CD4⁺ T cells with TNF resulted in no alteration in the phosphorylation of downstream mitogen-activated protein kinases compared with that in their *Cpdm*^{+/-} counterparts (Supplementary Fig. 6d). We also detected (by flow cytometry) a greater intensity of phosphorylation of ERK1/2 in *Cpdm*^{-/-} T_{reg} cells than in *Cpdm*^{+/-} T_{reg} cells after stimulation via the TCR (Fig. 5d) but not after stimulation with PMA plus ionomycin (Supplementary Fig. 6e). This 'hyper-activated' TCR signaling was 'rescued' by retroviral reconstitution of *Cpdm*^{-/-} CD4⁺ T cells with

wild-type SHARPIN (Fig. 5e). These results suggested that SHARPIN acted as an intrinsic negative regulator of TCR signaling.

Interaction of SHARPIN with the TCR ζ -Zap70 complex

We next investigated whether SHARPIN deficiency affected the endocytosis of TCRs. We found equivalent surface expression of TCR β on wild-type and *Cpdm*^{-/-} CD4⁺ T cells or T_{reg} cells under steady-state conditions (Fig. 6a) and a similar rate of downregulation of TCR expression after stimulation via the TCR (Fig. 6b). To delineate the molecular mechanism by which SHARPIN regulated the phosphorylation of TCR ζ , we first used an *in vitro* activation assay³⁰. Immunoprecipitation of TCR ζ revealed that endogenous SHARPIN was recruited to the TCR complex in an activation-dependent manner in Jurkat human T cells (Fig. 6c). A co-immunoprecipitation assay of 293T human embryonic kidney cells, assessed after treatment with pervanadate, showed that SHARPIN precipitated together with Zap70 and/or precipitated together with TCR ζ only in the presence of Zap70 (Fig. 6d). The TCR stimulation-induced interaction between Zap70 and SHARPIN was also observed in mouse wild-type CD4⁺ T cells (Fig. 6e).

SHARPIN has a ubiquitin-like (UBL) domain and a putative ubiquitin-binding NPL4 zinc-finger domain (NZF) in the carboxy-terminal region, which are required for the binding to HOIP and ubiquitin, respectively⁶. To determine the molecular requirement for SHARPIN in its association with the TCR complex, we immunoprecipitated TCR ζ together with wild-type SHARPIN and SHARPIN mutants with alterations in the active site of the UBL domain (I269A) or NZF domain (F354V), or with deletion of the amino terminus (N), NZF domain (NZF), UBL domain (UBL) or carboxyl terminus (C), in Jurkat T cells. Whereas wild-type SHARPIN and the I269A and N SHARPIN mutants precipitated together with TCR ζ chain, SHARPIN mutants with alterations or deletions of the NZF domain, including the F354V, NZF and C mutants, were unable to bind to the TCR ζ chain (Fig. 6f). However, Zap70 interacted with both wild-type SHARPIN and SHARPIN mutants with alterations in the UBL or NZF domain (I269A and F354V) (Fig. 6g). This suggested that the NZF domain of SHARPIN was required for binding to the TCR ζ chain in a ubiquitin-dependent manner. Collectively, these data indicated that SHARPIN bound to TCR ζ and the TCR ζ -Zap70 complex.

Impairment of the TCR ζ -Zap70 association SHARPIN ubiquitination impairs

We speculated that modification of SHARPIN by ubiquitin might be involved in the regulation of TCR signaling. We performed an *in vivo* ubiquitination assay and found that ubiquitination of wild-type SHARPIN was promoted by stimulation via the TCR and that ubiquitination was abolished in the F354V SHARPIN mutant but not in the I269A SHARPIN mutant (Fig. 7a), suggestive of NZF domain-dependent but HOIP-independent ubiquitination of SHARPIN. To investigate which lysine residue of SHARPIN or ubiquitin-chain linkage was responsible for the ubiquitination of SHARPIN, we generated Jurkat T cell lines that stably expressed FLAG-tagged SHARPIN and analyzed the endogenous ubiquitin modification of FLAG-tagged SHARPIN by mass spectrometry^{31,32}. Lys42, Lys168-Lys169 and Lys312 of SHARPIN were identified as the sites modified by ubiquitin, and ubiquitin chains were formed on SHARPIN via K11, K48 and K63 linkage (Fig. 7b). To

investigate the details of the ubiquitin-chain formation, we performed a ubiquitin assay with ubiquitin mutants retaining no lysine residues or only one lysine residue at position 11, 48 or 63 and found that all of these ubiquitin chains, but not a linear ubiquitin chain, were assembled on SHARPIN upon TCR stimulation (Fig. 7c). Next, to determine which lysine residue of SHARPIN was required for its ubiquitin-dependent function, we generated SHARPIN mutants with replacement of these lysine residues with arginine (K42R, K168–169R or K312R). When the K42R, K168–169R and K312R SHARPIN mutants were overexpressed in Jurkat T cells, ubiquitination of these was less than the ubiquitination of wild-type SHARPIN (Supplementary Fig. 7a), and among these, the K312R and F354V SHARPIN mutants were not efficiently conjugated with K63-linked ubiquitin chains (Fig. 7d and Supplementary Fig. 7b,c). In addition, using a K63-specific antibody, we detected endogenous formation of K63-linked ubiquitin chains on SHARPIN in mouse CD4⁺ T cells (Fig. 7e).

Because the K63-linked ubiquitin chain regulates signaling pathways³³, we hypothesized that K63-linked ubiquitination at Lys312 of SHARPIN might be involved in the negative regulation of TCR signaling. We assessed the effect of ubiquitination of SHARPIN on formation of the TCR complex. In Jurkat T cells, TCR ζ did not interact with the K312R or F354V SHARPIN mutant, whereas wild-type SHARPIN and the I269A SHARPIN mutant bound Zap70 (Fig. 7f); this indicated that the formation of K63-linked ubiquitin chains on SHARPIN was required for binding to TCR ζ . TCR stimulation–induced binding of TCR ζ to Zap70 in 293T cells was inhibited by the expression of wild-type SHARPIN but was not affected by expression of the F354V or K312R SHARPIN mutant (Fig. 7g). We then performed immunoprecipitation of endogenous Zap70 and observed greater interaction between TCR ζ and Zap70 in *Cpdm*^{-/-} CD4⁺ T cells than in wild-type CD4⁺ T cells (Fig. 7h). We assessed the interaction of TCR ζ mutants lacking individual ITAMs with wild-type SHARPIN in Jurkat T cells by co-immunoprecipitation and found that the interaction between TCR ζ and SHARPIN was dependent on the third ITAM of TCR ζ (Fig. 7i), which is known to be a docking site for Zap70 (ref. 34). Our results suggested that modification of SHARPIN by a K63-linked ubiquitin chain led to inhibition of the interaction between TCR ζ and Zap70 after stimulation via the TCR.

SHARPIN regulation of TCR signaling in T_{reg} cell generation

We next assessed the phosphorylation status of TCR signaling molecules in *Cpdm*^{-/-} CD4⁺ T cells retrovirally transduced with the F354V, K312R or I269A SHARPIN mutant. Unlike reconstitution with the I269A SHARPIN mutant, reconstitution with the F354V or K312R SHARPIN mutant did not rectify the increased phosphorylation of TCR ζ , Zap70 and ERK1/2 observed in *Cpdm*^{-/-} CD4⁺ T cells (Fig. 8a). To investigate whether the regulation of TCR signaling by K63-linked ubiquitination of SHARPIN led to the control of T_{reg} cell generation, we retrovirally transduced *Cpdm*^{-/-} CD4⁺ T cells with wild-type SHARPIN or the SHARPIN mutants noted above and induced T_{reg} cells via stimulation with TGF- β . Retroviral expression of the F354V or K312R SHARPIN mutant did not ‘rescue’ the reduced induction of Foxp3 expression observed in *Cpdm*^{-/-} CD4⁺ T cells (Fig. 8b). We next retrovirally expressed wild-type SHARPIN or the F354V, K312R or I269A SHARPIN mutant in *Cpdm*^{-/-} whole BM cells and transferred the cells into irradiated wild-type host

mice. We observed an almost complete ‘rescue’ of T_{reg} cell generation in both the thymus and spleen of host mice reconstituted with *Cpdm*^{-/-} donor cells transfected to express wild-type SHARPIN or the I269A SHARPIN mutant, while the generation of T_{reg} cells from untransduced donor cells was not affected (Fig. 8c–e). However, T_{reg} cell generation was not detected in hosts reconstituted with *Cpdm*^{-/-} BM cells transfected to express the F354V or K312R SHARPIN mutant (Fig. 8c–e). To investigate whether SHARPIN regulates TCR signaling and T_{reg} cell generation in a LUBAC-independent manner, we retrovirally transduced short hairpin RNA (shRNA) targeting SHARPIN or HOIP into wild-type total BM cells and transferred the cells into wild-type irradiated host mice. We observed impaired T_{reg} cell development in host mice reconstituted with progenitor cells transduced with SHARPIN-specific shRNA but not in host mice reconstituted with progenitor cells transduced with HOIP-specific shRNA (Supplementary Fig. 8). In addition, we noted greater phosphorylation of TCRζ and ERK1/2 in CD4⁺ T cells derived from progenitor cells transduced with SHARPIN-specific shRNA than in those derived from progenitor cells transduced with nontargeting control shRNA or HOIP-specific shRNA (Supplementary Fig. 8). Collectively, these results suggested that SHARPIN controlled T_{reg} cell development by negatively regulating TCR signaling.

DISCUSSION

Here we demonstrated a critical role for SHARPIN in the development and function of T_{reg} cells; this role explained the autoinflammatory phenotype of SHARPIN-deficient mice. Several mechanisms have been proposed to explain how SHARPIN deficiency leads to skin and systemic multi-organ inflammation. Increased apoptosis, potentially by aberrant regulation of NF-κB, in various cell types is regarded as the main cause of dermatitis^{5–7,10,35}. Augmented T_H2 cytokine-mediated accumulation of eosinophils in the skin and lungs may or may not contribute to the inflammation of *Cpdm*^{-/-} mice^{36,37}. Moreover, SHARPIN might acquire tissue-specific roles, since the organs affected are not all identical in *Cpdm*^{-/-} mice³⁸. Consistent with that, systemic inflammation, except for dermatitis, is substantially alleviated by the deficiency in mature B cells and T cells in *Rag1*^{-/-}*Cpdm*^{-/-} mice¹⁴. We demonstrated a significant reduction in the development and function of T_{reg} cells in *Cpdm*^{-/-} mice, and reconstitution of *Cpdm*^{-/-} neonatal mice with SHARPIN-sufficient T_{reg} cells markedly alleviated their inflammation responses. Thus, our study highlights a previously unknown mechanism for SHARPIN in the autoinflammatory responses via its control of T_{reg} cells.

Although TCR signaling is essentially required for Foxp3 expression and the acquisition of suppressive activity by T_{reg} cells^{20,39}, the strength and duration of TCR signaling, which are modulated by the quantity of phosphorylation of TCR-CD3 ITAMs, also have qualitatively different effects on the development and effector function of T cells and T_{reg} cells^{40,41}. Indeed, signaling-deficient TCRζ is linked to enhanced development and suppressive function of T_{reg} cells via selective regulation of downstream signaling responses²⁶. Our observations of SHARPIN in T_{reg} cells can be explained by a selection-shift model⁴². In this model, fewer thymocytes reach the threshold for negative selection (selection shift) by attenuation of TCR signaling than with intact TCR signaling and, as a consequence, self-reactive T cells are less negatively selected^{25,42}. In this context, the augmented TCR

signaling in *Cpdm*^{-/-} thymocytes might result in a selection shift toward lower self-reactivity through the elimination of more self-reactive T cells that would undergo more apoptosis, which would lead to a reduced number of T_{reg} cells. The enhanced apoptosis of thymic *Cpdm*^{-/-} T_{reg} cells and the more naive-like phenotype of peripheral *Cpdm*^{-/-} T cells in the mixed-BM chimeras would support our hypothesis. Therefore, we have demonstrated a previously unidentified role for SHARPIN in regulating TCR signaling and T_{reg} cell development.

In addition, SHARPIN deficiency led to robust production of IL-17. Although published studies have reported the production of large amounts of T_H2 cytokines in skin cells or splenocytes from *Cpdm*^{-/-} mice^{14,36}, we found that SHARPIN specifically regulated IL-17 production by T_{reg} cells in a cell-intrinsic manner. The T_H17-skewed response of *Cpdm*^{-/-} T_{reg} cells might have been due to increased TCR signaling that inhibited the induction of Foxp3 expression and favored T_H17 differentiation through the activation of the PI(3)K-Akt-mTOR kinase pathway^{43,44}. Indeed, we observed augmented phosphorylation of Akt and Foxo1 in *Cpdm*^{-/-} T cells, which prompted the *Cpdm*^{-/-} T_{reg} cells to acquire a T_H17-like phenotype and led to the impaired suppressive function.

Another notable finding of our study was that SHARPIN inhibited the interaction between TCR ζ and Zap70, in a ubiquitin-dependent manner, after ligation of the TCR. Increased binding of Zap70 to TCR ζ , which prevents dephosphorylation of TCR ζ ⁴⁵, probably led to the increased phosphorylation of TCR ζ in *Cpdm*^{-/-} T cells. Although our study showed that K63-linked ubiquitination of SHARPIN was critical for binding to TCR ζ and subsequent inhibition of the TCR ζ -Zap70 interaction, it is still unclear how SHARPIN affected the association of Zap70. The binding of SHARPIN to TCR ζ via the K63-linked ubiquitin chain might structurally interfere with the binding of Zap70. Another possibility is that ubiquitinated SHARPIN might recruit other regulators, such as Cbl-b and Itch, which catalyze the conjugation of K33-linked polyubiquitin chains to TCR ζ to abrogate the binding of Zap70 (ref. 31). Further biochemical and proteomics approaches will be needed to clarify the precise mechanism of the SHARPIN-mediated regulation of TCR ζ .

ONLINE METHODS

Mice

C57BL/6 SHARPIN-deficient (*Cpdm*^{-/-}), C57BL/6, B6.SJL (CD45.1⁺ congenic) and *Rag1*^{-/-} mice were obtained from the Jackson Laboratory. *Cpdm*^{-/-} mice were bred with *Foxp3*^{YFP^{Cre} mice to mark T_{reg} cells with the YFP reporter. For the generation of mice with transgenic TCR expression, OT-II mice (with transgenic expression of a TCR specific for ovalbumin peptide of amino acids 323–339) were crossed with *Cpdm*^{-/-} mice. All animal protocols were approved by members of the Institutional Animal Care and Use Committee of the La Jolla Institute for Allergy and Immunology. Age-matched both female and male mice were used in experiments. Wherever possible, pilot experiments were performed to determine requirements for sample size. Exclusion criteria such as scant staining or low cell yield due to technical issues were pre-established. A blinding method was used to assess the outcome. No randomization was used.}

Flow cytometry

The fluorochrome-labeled antibodies for flow cytometry are listed in Supplementary Table 1. For intracellular staining, cells were stained with antibodies to surface markers and then fixed and permeabilized with Cytofix/Cytoperm buffer (BD Biosciences), followed by staining with anti-Foxp3, anti-CTLA4, anti-IL-5, anti-IL-13, anti-IL-17 or anti-IFN- γ diluted in Perm/Wash buffer (BD Biosciences). For intracellular staining of phosphorylated ERK1/2 or p65, cells were fixed with Cytofix Fixation buffer (BD Biosciences) for 10 min at 37 °C and then permeabilized in 90% methanol for 30 min at 4 °C. Cells were then washed twice in Stain buffer (BD Biosciences) and stained with antibody to phosphorylated ERK1/2 or p65. Acquisition was performed on a FACSCantoII flow cytometer (BD Biosciences) and analyzed with FlowJo software (version 9.7.6; Tree Star).

In vivo induction of T_{reg} cells

Sorted naive CD4⁺CD62L⁺CD44⁻CD25⁻ T cells from *Cpdm*^{+/-} or *Cpdm*^{-/-} OT-II mice were isolated by cell sorting and 1×10^6 cells were retro-orbitally injected into wild-type B6 CD45.1⁺ congenic mice. The next day, the recipient mice were immunized with ovalbumin peptide (amino acids 323–339) (10 μ g; AnaSpec). 5 d after administration of that ovalbumin peptide, cells were collected from the spleen and peripheral (axillary, branchial and inguinal) lymph nodes. Foxp3 expression was analyzed by flow cytometry.

In vitro T_{reg} cell generation

Sorted naive CD4⁺CD62L⁺CD25⁻ T cells (2×10^5) from *Cpdm*^{+/-} or *Cpdm*^{-/-} mice were stimulated for 3 d in 96-well flat-bottomed plates with plate-bound anti-CD3 (2C11; Biolegend), soluble anti-CD28 (37.5; Bio-Xcell) and human IL-2 (PeproTech), together with the appropriate concentrations of recombinant human TGF- β (PeproTech).

In vitro T_{reg} cell suppression assay

For the *in vitro* suppression assay, sorted naive CD4⁺CD62L⁺CD25⁻ T cells (5×10^4 cells) were labeled with 5 μ M Violet (Invitrogen) for 10 min at 37 °C in PBS and 0.1% BSA and were then co-cultured with sorted CD4⁺CD25⁺YFP⁺ T_{reg} cells (5×10^4 cells) from *Foxp3*^{YFPCre} *Cpdm*^{+/-} and *Foxp3*^{YFPCre} *Cpdm*^{-/-} mice in the presence of splenocytes (1×10^5) that had been depleted of T cells, plus soluble anti-CD3 (identified above). 4 d later, cells were harvested, and violet dilution was measured by flow cytometry.

Colitis induction

Sorted naive CD4⁺CD25⁻CD45RB^{hi} T cells (4×10^5 cells per mouse) from wild-type CD45.1⁺ congenic mice were retro-orbitally injected into C57BL/6 *Rag1*^{-/-} mice together with sorted CD4⁺CD25⁺YFP⁺ T_{reg} cells (1×10^5 cells per mouse) from *Foxp3*^{YFPCre} *Cpdm*^{+/-} or *Foxp3*^{YFPCre} *Cpdm*^{-/-} mice. The recipient *Rag1*^{-/-} mice were monitored weekly for 8 weeks.

Multi-cytokine assay

For measurement of the level of multi-cytokine production, sorted CD4⁺CD25⁺YFP⁺ T cells were stimulated with plate-bound anti-CD3 (2C11; Bio-legend) and soluble anti-CD28

(37.5; Bio-legend). After 36 h, cytokines were detected in the culture supernatants using the Bio-Plex Pro Mouse Cytokine 23-plex Assay (Bio-Rad) according to the manufacturer's instructions.

***In vitro* activation**

Jurkat T cells were transiently transfected by electroporation (240V, 960 mF; Bio-Rad) to express FLAG-tagged TCR ζ or HA-tagged Zap70 and Myc-tagged SHARPIN. After 48 h, cells were resuspended in hypotonic buffer (20 mM HEPES-KOH, 10 mM KCl, 1 mM MgCl₂, 1 mM EDTA, 1 mM EGTA, 1 mM DTT, 50 mM NaF, 2 mM Na₃VO₄, and 10 μ g/ml each of aprotinin and leupeptin) and were lysed by freezing and thawing. Cell lysates were incubated at 37 °C for the appropriate times or were left untreated on ice. After incubation, an equivalent volume of hypotonic buffer containing 0.1% NP40 and 300 mM NaCl was added. The lysates were incubated overnight with anti-FLAG M2 agarose beads (Sigma) at 4 °C.

Immunoblot analysis and immunoprecipitation

The antibodies for immunoblot analysis are listed in Supplementary Table 1. Cells were lysed with NP-40 lysis buffer (1% NP-40, 20 mM Tris-HCl, pH 7.5, 150 mM NaCl, 5 mM EDTA, 50 mM NaF, 2 mM Na₃VO₄, and 10 μ g/ml each of aprotinin and leupeptin) or were lysed with 1 $\times$ SDS sample buffer (50 mM Tris-HCl, pH 6.8, 100 mM DTT, 2% SDS, and 10% glycerol). Cell lysates were adjusted to 0.1% NP-40 and incubated with anti-FLAG M2 agarose beads (Sigma) overnight at 4 °C. The immunocomplexes were recovered by low-speed centrifugation, and the beads were washed extensively with the binding buffer with 0.1% NP-40 and then were eluted with buffer containing 20 mM Tris-HCl (pH 8.0) and 2% SDS.

For detection of the ubiquitinated form of SHARPIN, Jurkat T cells stably expressing triple-FLAG-tagged SHARPIN were lysed with 1% SDS in TBS (50 mM Tris, pH 7.5 and 150 mM NaCl) supplemented with 10 mM N-ethylmaleimide, then were incubated at 95 °C for 10 min and then diluted to 0.1% SDS with 1% NP-40 containing TBS. FLAG-tagged SHARPIN proteins were immunoprecipitated through the use of anti-FLAG M2 agarose (Sigma-Aldrich) for 4 h at 4 °C. The immunoprecipitated proteins were eluted by 2% SDS in 50 mM Tris (pH 7.5), and these eluates were again diluted to 0.1% SDS with 1% NP-40 containing TBS, followed by a second immunoprecipitation using anti-FLAG-M2 agarose. The immunoprecipitates were eluted with triple-FLAG peptide. The eluates were subjected to immunoblot analysis and mass spectrometry.

Immunoprecipitated proteins were separated by SDS-PAGE and transferred to nitrocellulose membrane (Bio-Rad). Membranes were visualized by immunoblot analysis with the enhanced chemiluminescence detection system (ECL; GE Healthcare). When necessary, membranes were stripped by incubation in stripping buffer (Thermo Fisher Scientific) for 15 min with constant agitation and washed, and then were reprobed with various other antibodies.

Retroviral transduction and BM reconstitution

For construction of vectors encoding SHARPIN- or HOIP-specific shRNA, oligonucleotides were cloned in the LMP vector according to the manufacturer's protocol (Open Biosystems). Oligonucleotide sequences are listed in Supplementary Table 2. For the generating of BM chimeric mice expressing retroviral constructs, Plat-E cells were transfected with 3 µg of pMIG or LMP vector with 9 µl of TransIT-LT1 (Mirus). At 48 h, the culture supernatant containing retrovirus was collected. BM from *Cpdm^{+/-}* and *Cpdm^{-/-}* mice was depleted of mature T cells and then cultured for 24 h in IL-3 (10 ng/ml), IL-6 (10 ng/ml) and SCF (100 ng/ml) (all from Peprotech) containing complete DMEM before initial retroviral infection, and the cells were infected with retrovirus together with 5 µg/ml polybrene by centrifugation at 2,000 rpm for 60 min at room temperature. 2 d after infection, retrovirally transduced bone marrow cells were injected into lethally irradiated (900 rads) SJL (CD45.1⁺ congenic) recipient mice. Recipient mice were euthanized and analyzed 8 weeks after reconstitution.

Statistics

Statistical analyses were performed using a two-tailed, unpaired Student's *t*-test. A *P* value of less than 0.05 was considered statistically significant.

Acknowledgments

We thank A. Rudensky (Memorial Sloan Kettering Cancer Center) for *Foxp3^{YFPCre}* mice. Supported by the National Institute of Allergy and Infectious Diseases of the US National Institutes of Health (RO1AI62969, RO1AI78272 and PO1AI089624).

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Figure 1.

Enhanced T cell activation and spontaneous multi-organ inflammation in *Cpdm*^{-/-} mice. **(a)** Hematoxylin-and-eosin (H&E) staining (left) and histology scores (right) of tissue sections of the lungs and small intestine (SI) of *Cpdm*^{+/-} and *Cpdm*^{-/-} mice ($n = 8$ per group). Original magnification, ×100. **(b)** Total CD4⁺ T cells in the lungs of *Cpdm*^{+/-} and *Cpdm*^{-/-} mice ($n = 6$ per group). **(c)** Flow cytometry analyzing the expression of CD62L and CD44 in CD4⁺ T cells from the lungs of *Cpdm*^{+/-} and *Cpdm*^{-/-} mice ($n = 6-7$ per group) (above), and frequency of CD62L⁺ or CD44⁺ cells among those CD4⁺ T cells (below). Numbers adjacent to outlined areas (above) indicate percent CD62L⁺CD44⁻ cells (top left) or CD62L⁻CD44⁺ cells (bottom right). **(d)** Apoptosis of naive CD4⁺CD62L⁺CD44⁻CD25⁻ T cells obtained from *Cpdm*^{+/-} or *Cpdm*^{-/-} mice ($n = 6$ per group) and left unstimulated (US) or stimulated with anti-CD3 (CD3) or with anti-CD3 plus anti-CD28 (CD3+CD28). **(e)** Flow cytometry analyzing the proliferation of CD4⁺ T cells from the spleen of *Cpdm*^{+/-} and *Cpdm*^{-/-} mice ($n = 6$ per group) with or without stimulation with anti-CD3 and anti-CD28, stained with CellTrace Violet. **(f)** Flow cytometry analyzing cytokines (above plots) in CD4⁺ T cells isolated from the lungs of *Cpdm*^{+/-} and *Cpdm*^{-/-} mice ($n = 4-6$ per group) and then stimulated with PMA plus ionomycin (above), and frequency of cytokine-expressing cells among those CD4⁺ T cells (below). Numbers adjacent to outlined areas (above) indicate percent cytokine-positive CD4⁺ T cells. Each symbol (**c,f**) represents an individual mouse; small horizontal lines indicate the mean (± s.d.). NS, not significant; * $P < 0.01$, ** $P < 0.001$.

and *** $P < 0.0001$ (two-tailed unpaired t -test). Data are pooled from or representative of two to four independent experiments (mean and s.d. in **a,b,d**).

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Figure 2.

$Cpdm^{-/-}$ mice exhibit impaired generation of T_{reg} cells. **(a)** Flow cytometry of cells from the thymus (Thy), spleen (SP), lymph nodes (LN), mesenteric lymph nodes (MLN), colon, small intestine (SI) and lungs of 4-week-old $Cpdm^{+/-}$ and $Cpdm^{-/-}$ mice ($n = 7-12$ per group), analyzing the expression of Fopx3 and CD4 (left), and frequency of Fopx3⁺CD4⁺ T_{reg} cells in those tissues (right). Numbers adjacent to outlined areas (left) indicate percent Fopx3⁺CD4⁺ (T_{reg}) cells. **(b)** Total Fopx3⁺CD4⁺ cells in the thymus, spleen, lymph nodes and mesenteric lymph nodes of $Cpdm^{+/-}$ and $Cpdm^{-/-}$ mice ($n = 8$ per group). **(c)** Flow cytometry (as in **a**) of pregated CD45.1⁺ or CD45.2⁺ cells from the thymus and spleen of host mice (CD45.1⁺) ($n = 6-9$ per group) reconstituted with $Cpdm^{+/-}$ (CD45.2⁺) BM cells or $Cpdm^{-/-}$ (CD45.2⁺) BM cells (BMC) or with a mixture of wild-type (CD45.1⁺) BM cells

(WT) and *Cpdm*^{-/-} (CD45.2⁺) BM cells (Mixed) (above), and frequency of Foxp3⁺CD4⁺ T_{reg} cells in those tissues (below). **(d,e)** Flow cytometry analyzing Foxp3 expression in naive CD4⁺CD62L⁺CD44⁻CD25⁻ T cells obtained from *Cpdm*^{+/-} and *Cpdm*^{-/-} mice and stimulated with various concentrations (above plots) of anti-CD3 (0.5, 1 or 3 µg/ml) plus anti-CD28 (1 µg/ml), and TGF-β (1 ng/ml) **(d)**, or with anti-CD3 (2 µg/ml) plus anti-CD28 (1 µg/ml) and various concentrations (above plots) of TGF-β (0.5, 1 or 2.5 µg/ml). Numbers above bracketed lines indicate percent Foxp3⁺ cells. **(f)** Flow cytometry of cells from the spleen and lymph nodes of congenic B6 (CD45.1⁺) host mice (*n* = 4 per group) that received adoptively transferred naive CD4⁺CD62L⁺CD44⁻CD25⁻ T cells from *Cpdm*^{-/-} and *Cpdm*^{+/-} (CD45.2⁺) OT-II mice (which have transgenic expression of a TCR specific for ovalbumin peptide), followed by immunization of the host mice with that ovalbumin peptide (10 µg), analyzing the expression of Foxp3 and CD45.2 (left), and frequency of CD45.2⁺ T_{reg} cells in those tissues (right). Numbers adjacent to outlined areas (left) indicate percent Foxp3⁺ (T_{reg}) CD45.2⁺ (donor) cells. Each symbol **(a,f)** represents an individual mouse; small horizontal lines indicate the mean (± s.d.). **P* < 0.05, ***P* < 0.01, ****P* < 0.001 and *****P* < 0.0001 (two-tailed unpaired *t*-test). Data are pooled from or representative of two to four independent experiments (mean and s.d. in **b**).

Figure 3.

SHARPIN-deficient T_{reg} cells fail to retain their suppressive activity and Foxp3 expression.

(a) Flow cytometry analyzing the expression of surface markers (above plots) on CD4⁺Foxp3⁺ T_{reg} cells from *Cpdm*^{+/-} and *Cpdm*^{-/-} mice (*n* = 6 per group) (above), and frequency of marker-expressing cells among T_{reg} cells in those mice (below). (b) Flow cytometry analyzing the expression of Foxp3 and YFP by CD4⁺ T_{reg} cells from *Foxp3*^{YFP/Cre}*Cpdm*^{+/-} and *Foxp3*^{YFP/Cre}*Cpdm*^{-/-} mice (*n* = 6 per group) (left and middle) or by CD4⁺Foxp3⁺YFP⁺ T_{reg} cells sorted from a *Foxp3*^{YFP/Cre}*Cpdm*^{-/-} mouse (right).

Numbers adjacent to outlined areas indicate percent Foxp3⁺YFP⁺ cells. **(c)** Division of naive CD4⁺CD62L⁺CD25⁺ T cells stimulated with anti-CD3 plus irradiated splenocytes (after depletion of T cells) alone (far left) or in the presence of T_{reg} cells sorted from Foxp3^{YFP}Cre Cpdn^{+/-} or Foxp3^{YFP}Cre Cpdn^{-/-} mice, at various ratios of T_{reg} cells to naive T cells (above plots) (middle and right). **(d)** Weight of Rag1^{-/-} host mice ($n = 6-12$ per group) at various times after adoptive transfer of CD4⁺CD45RB^{hi} (CD45.1⁺) T cells alone (None) or together with CD4⁺CD25⁺YFP⁺ (CD45.2⁺) T_{reg} cells from Foxp3^{YFP}Cre Cpdn^{+/-} or Foxp3^{YFP}Cre Cpdn^{-/-} mice, presented relative to weight at time 0, set as 1. **(e)** H&E staining (above) and histology scores (below) of colon tissues from the Rag1^{-/-} host mice in **d** ($n = 6-10$ per group) at 8 weeks after cell transfer. Original magnification (above), $\times 100$. **(f)** Flow cytometry analyzing Foxp3 expression in pre-gated CD45.2⁺CD4⁺ T cells in various tissues (above plots) from the mice in **e** (above), and frequency of CD45.2⁺ Foxp3⁺ T cells in those tissues (below). **(g)** Multiplex assay of the production of various cytokines (horizontal axis) in Cpdn^{+/-} and Cpdn^{-/-} CD4⁺CD25⁺YFP⁺ T_{reg} cells stimulated with anti-CD3 plus anti-CD28. **(h)** Flow cytometry analyzing the expression of IL-17 and Foxp3 in CD4⁺Foxp3⁺ T_{reg} cells isolated from spleen or lungs of Cpdn^{+/-} and Cpdn^{-/-} mice ($n = 5$ per group) and frequency of IL-17⁺ T_{reg} cells in those tissues (right). Numbers adjacent to outlined areas indicate percent IL-17⁺ Foxp3⁺ (T_{reg}) cells. Each symbol (**f,h**) represents an individual mouse; small horizontal lines indicate the mean ($\pm$ s.d.). * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ and **** $P < 0.0001$ (two-tailed unpaired t -test). Data are pooled from or representative of two to four independent experiments (mean and s.d. in **a,d,e,g**).

Figure 4.

Adoptive transfer of wild-type T_{reg} cells reduces the severity of systemic inflammation in neonatal *Cpdm*^{-/-} mice. **(a)** H&E staining (above) and histology scores (below) of tissue sections of the lungs and small intestine of *Cpdm*^{+/-} and *Cpdm*^{-/-} mice ($n = 6$ per group) that received, at 1 d of age (neonatal period), either no cells (left half) or CD4⁺CD25⁺YFP⁺ T_{reg} cells (5×10^5) sorted from *Foxp3*^{YFPCre} *Cpdm*^{+/+} mice (right half), analyzed 6 weeks after cell transfer. Original magnification (above), $\times 100$. **(b)** Total eosinophils (Eos), lymphocytes (Lym) and monocytes (Mon) in the lungs of mice as in **a** ($n = 6$ per group). **(c)** Flow cytometry of CD4⁺ T cells isolated from the lungs of mice as in **a** ($n = 5-6$ per group), analyzing the expression of CD62L and CD44 (above), and frequency of CD62L⁺ or CD44⁺ cells among those CD4⁺ T cells (below). Numbers adjacent to outlined areas (above) indicate percent CD62L⁺CD44⁻ cells (top left) or CD62L⁻CD44⁺ cells (bottom right). **(d)** Flow cytometry of CD4⁺ T cells isolated from lungs of the mice in **c**, analyzing the expression of IL-17, IL-5 or IL-13 (left), and frequency of IL-17⁺, IL-5⁺ or IL-13⁺ cells among those CD4⁺ T cells (right). Numbers adjacent to outlined areas (left) indicate percent IL-17⁺ cells (top row), IL-5⁺ cells (middle row) or IL-13⁺ cells (bottom row). Each symbol (**c,d**) represents an individual mouse; small horizontal lines indicate the mean ($\pm$ s.d.). * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ and **** $P < 0.0001$ (two-tailed unpaired t -test). Data are pooled from or representative of three independent experiments (mean and s.d. in **a,b**).

Figure 5.

SHARPIN acts as a negative regulator of TCR signaling. **(a)** Immunoblot analysis of total and phosphorylated (p-) IκBα in *Cpdm*^{+/−} and *Cpdm*^{−/−} CD4⁺ T cells stimulated for various times (above lanes) with anti-CD3 plus anti-CD28 (CD3⁺CD28), PMA plus ionomycin (PMA+Iono) or TNF; Grb2 serves as a loading control throughout. **(b)** Flow cytometry analyzing the intracellular staining of phosphorylated p65 in CD4⁺ T cells sorted from *Cpdm*^{+/−} and *Cpdm*^{−/−} mice and left unstimulated (US) or stimulated for 15 min (Stim) as in a (right, overlay of left and middle plots). **(c)** Immunoblot analysis of SHARPIN and total and phosphorylated TCRζ, Zap70, LAT, ERK1/2, JNK1/2, Lck and PLCγ in *Cpdm*^{+/−} and *Cpdm*^{−/−} CD4⁺ T cells stimulated for various times (above lanes) with anti-CD3. **(d)** Immunoblot analysis of SHARPIN and phosphorylated TCRζ and ERK1/2 in *Cpdm*^{+/−} and *Cpdm*^{−/−} T_{reg} cells stimulated for various times (above lanes) with anti-CD3 (above), and flow cytometry analyzing phosphorylated ERK1/2 in *Cpdm*^{+/−} and *Cpdm*^{−/−} CD4⁺Foxp3⁺ T cells or CD4⁺Foxp3[−] T_{reg} cells (left margin) left unstimulated or stimulated with anti-CD3 (below; right, as in **b**)). **(e)** Immunoblot analysis of SHARPIN and phosphorylated TCRζ, Zap70 and ERK1/2 in GFP⁺CD4⁺ T cells sorted from *Cpdm*^{+/−} and *Cpdm*^{−/−} splenocytes activated with concanavalin A and retrovirally transduced with empty vector expressing green fluorescent protein (GFP) alone (EV) or vector expressing GFP plus wild-type

SHARPIN (WT), followed by stimulation for various times (above lanes) with anti-CD3.
Data are representative of three independent experiments.

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Figure 6.

Zap70-mediated interaction between TCRζ and SHARPIN after stimulation via the TCR. **(a)** Flow cytometry analyzing the cell-surface expression of TCRβ on pre-gated CD4⁺Foxp3⁻ T cells (left) or CD4⁺Foxp3⁺ T_{reg} cells (right) from *Cpdm*^{+/-} and *Cpdm*^{-/-} mice (*n* = 6 per group). **(b)** Downregulation of TCRβ expression in CD4⁺ T cells obtained from *Cpdm*^{+/-} and *Cpdm*^{-/-} mice (*n* = 4 per group) and treated with cycloheximide (50 nM) and anti-CD3. **(c)** Immunoblot analysis of FLAG-tagged TCRζ and other components of the TCR signaling complex (left margin) in Jurkat T cells transfected to express FLAG-tagged TCRζ, followed by hypertonic cell lysis for 0 or 5 min (above lanes), assessed in total lysates (left half) or in lysates after immunoprecipitation (IP) with anti-FLAG (α-FLAG). **(d)** Immunoblot analysis (IB) of the immunoprecipitation of FLAG-tagged TCRζ or hemagglutinin (HA)-tagged Zap70 together with Myc-tagged SHARPIN in 293T cells transfected to express various combinations of those tagged proteins (above lanes) and stimulated (+) or not (–) with pervanadate (PV) (top group), and immunoblot analysis of lysates without immunoprecipitation (bottom group). **(e)** Immunoblot analysis of TCRζ and SHARPIN immunoprecipitated together with endogenous Zap70 (via anti-Zap70), in mouse CD4⁺ T cells stimulated for 0–10 min (above lanes) with anti-CD3 (right), and immunoblot analysis (with anti-Zap70, anti-TCRζ or anti-SHARPIN) of lysates without immunoprecipitation (left half). IgG, lysates immunoprecipitated with immunoglobulin G (control). **(f)** Immunoblot analysis of the immunoprecipitation of FLAG-tagged TCRζ together with wild-type or mutant SHARPIN in Jurkat T cells transfected to express empty vector or wild-type SHARPIN or the I269A, F354V, N, NZF, UBL or C SHARPIN mutant (above), and stimulated with OKT3 or not (right half). **(g)** Immunoblot analysis of the immunoprecipitation of HA-tagged Zap70 with Myc-tagged wild-type or mutant SHARPIN in Jurkat T cells transfected to express empty vector or wild-type SHARPIN or the F354V or I269A SHARPIN mutant and stimulated with OKT3 or not (right half). Left half (**f,g**),

immunoblot analysis of lysates without immunoprecipitation. Data are pooled from or representative of two or three independent experiments.

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Figure 7.

SHARPIN inhibits the interaction between TCR ζ and Zap70 in a K63-linked ubiquitin chain-dependent manner. **(a)** Immunoblot analysis of the *in vivo* ubiquitination of Myc-tagged wild-type or mutant SHARPIN with HA-tagged ubiquitin (Ub) in Jurkat T cells transfected to express wild-type SHARPIN or the F354V or I269A SHARPIN mutant (above), and stimulated with OKT3 or not (right half), and immunoblot analysis of lysates without immunoprecipitation (left half). Immunoblot analysis of SHARPIN (bottom) serves as a control throughout. **(b)** Immunoblot analysis of the *in vivo* ubiquitination of FLAG-tagged wild-type SHARPIN with endogenous ubiquitin in Jurkat T cells stimulated with OKT3. **(c)** Immunoblot analysis of the *in vivo* ubiquitination of Myc-tagged wild-type SHARPIN with HA-tagged wild-type or mutant ubiquitin in Jurkat T cells transfected to express wild-type ubiquitin or a ubiquitin mutant retaining no lysine residues (Ub K^{gg}) or only one lysine residue at position 11 (Ub K11), 48 (Ub K48) or 63 (Ub K63), or no ubiquitin (Ub KO), and stimulated with OKT3 (right half). **(d)** Immunoblot analysis of the *in vivo* ubiquitination of Myc-tagged wild-type or mutant SHARPIN with K63-linked HA-tagged ubiquitin (HA-UbK63) mutant in Jurkat T cells transfected as in **a** and stimulated with OKT3. **(e)** Immunoblot analysis of the *in vivo* K63-linked ubiquitination (Ub K63) of endogenous SHARPIN, and of total SHARPIN (bottom), in wild-type mouse CD4⁺ T cells stimulated for various times (above lanes) with anti-CD3 (right half). **(f)** Immunoblot analysis of the immunoprecipitation of FLAG-tagged TCR ζ or HA-tagged Zap70 together with Myc-tagged wild-type or mutant SHARPIN in Jurkat T cells transfected to express wild-type SHARPIN or F354V, I269A or K312R mutant SHARPIN and stimulated with

OKT3 (right half). **(g)** Immunoblot analysis of the immunoprecipitation of HA-tagged Zap70 together with FLAG-tagged TCR ζ and Myc-tagged wild-type or mutant SHARPIN in 293T cells transfected as in a and stimulated with pervanadate or not (top group); below, immunoblot analysis of lysates without immunoprecipitation. **(h)** Immunoblot analysis of the immunoprecipitation of endogenous Zap70 (with anti-Zap70) in *Cpdm*^{+/-} and *Cpdm*^{-/-} mouse CD4⁺ T cells stimulated for various times (above lanes) with anti-CD3 (right half). **(i)** Immunoblot analysis of Xpress-tagged SHARPIN immunoprecipitated together with Myc-tagged wild-type or mutant TCR ζ in Jurkat T cells transfected to express Myc-tagged wild-type TCR ζ or the Y83F, Y110F, Y152F or Y83F–Y110F–Y152F TCR ζ mutant and stimulated with OKT3 (right half). Left two lanes (of each half), cells transfected to express Myc-tagged TCR ζ only (far left) or plus Xpress-tagged SHARPIN (second lane). Left half **(b, d, e, f, h, i)**, immunoblot analysis of lysates without immunoprecipitation. Data are representative of three independent experiments.

Figure 8.

Regulation of TCR signaling by SHARPIN is required for the generation of T_{reg} cells. **(a)** Immunoblot analysis of phosphorylated TCR ζ , Zap70 and ERK1/2, and total SHARPIN in GFP⁺CD4⁺ T cells sorted from *Cpdm*^{-/-} splenocytes activated with concanavalin A and retrovirally transduced with empty vector or vector expressing the F354V, K312R or I269A SHARPIN mutant, assessed after stimulation with anti-CD3. **(b)** Flow cytometry analyzing Foxp3 in *Cpdm*^{+/-} and *Cpdm*^{-/-} naive CD4⁺CD62L⁺CD44⁻CD25⁻ T cells stimulated with anti-CD3 plus anti-CD28, followed by retroviral transduction with empty vector or vector expressing or vector expressing wild-type SHARPIN or the F354V, I269A, K42R, K168-169R or K312R SHARPIN mutant (above plots) and, 1 d after transduction, cultured for 3 d in the presence of TGF- β (1 ng/ml) and IL-2. Numbers above bracketed lines indicate

percent Foxp3⁺ cells. (c) Flow cytometry analyzing GFP expression in cells from the peripheral blood of a congenic B6 (CD45.1⁺) host mouse 8 weeks after reconstitution with *Cpdm*^{-/-} BM cells transduced with vector expressing GFP alone. Numbers adjacent to outlined areas indicate percent GFP (untransduced) cells (left) or GFP⁺ (transduced) cells (right) among CD4⁺ T cells. (d,e) Flow cytometry analyzing Foxp3 and GFP in pregated GFPCD45.2⁺CD4⁺ (top row) or GFP⁺CD45.2⁺CD4⁺ (bottom row) T cells from the thymus (d) or spleen (e) of congenic B6 (CD45.1⁺) host mice ($n = 5-6$ per group) reconstituted with *Cpdm*^{+/-} or *Cpdm*^{-/-} BM cells transduced with empty vector expressing GFP alone (EV) or vector expressing GFP plus wild-type SHARPIN or the F354V, I269A or K312R SHARPIN mutant (right), and frequency of GFP⁺ T_{reg} cells (left). Numbers adjacent to outlined areas (left) indicate percent Foxp3⁺ (T_{reg}) cells among the pregated T cells. Each symbol (d,e) represents an individual mouse; small horizontal lines indicate the mean ($\pm$ s.d.). * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ and **** $P < 0.0001$ (two-tailed unpaired t -test). Data are pooled from or representative of two or three independent experiments (mean and s.d.).