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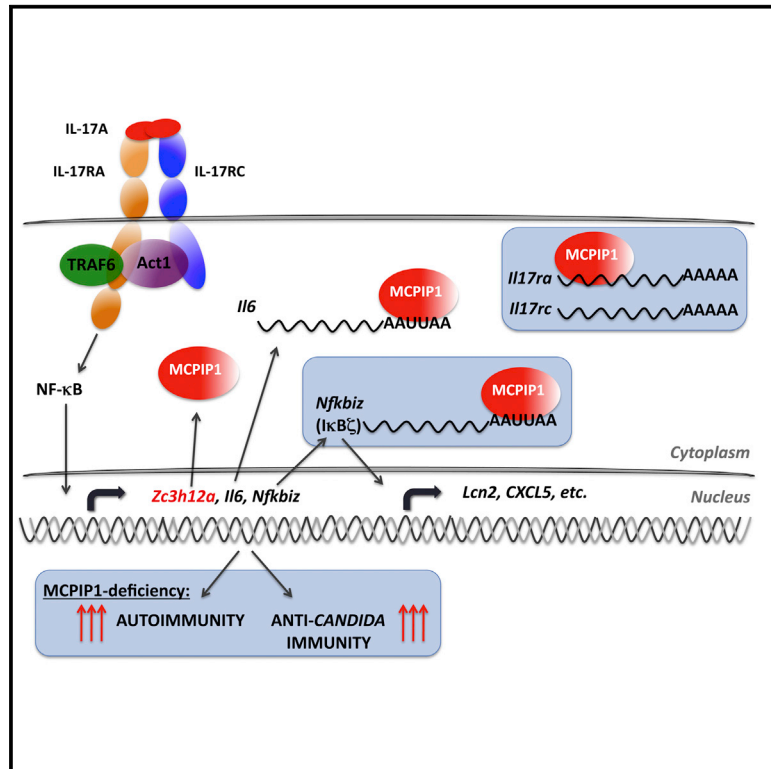
MCPIP1 Endoribonuclease Activity Negatively Regulates Interleukin-17-Mediated Signaling and Inflammation

Graphical Abstract

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In Brief

Interleukin-17 (IL-17) induces pathology in autoimmunity and infections and therefore its activity must be tightly regulated. Gaffen and colleagues report that the endoribonuclease MCPIP1 degrades IL-17-induced transcripts, including *Il6* and *Nfkbiz* (IκBζ), to negatively regulate IL-17 receptor signaling both in fungal immunity and experimental autoimmune encephalomyelitis.

Highlights

- MCPIP1 (Regnase-1) is a feedback inhibitor of IL-17 signal transduction

- MCPIP1 deficiency enhances immunity to fungi but exacerbates pathology in EAE

- MCPIP1 impairs activation of certain IL-17 target promoters by degrading IκBζ mRNA

- MCPIP1 degrades transcripts encoding IL-17R subunits independently of the 3′ UTR

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MCPIP1 Endoribonuclease Activity Negatively Regulates Interleukin-17-Mediated Signaling and Inflammation

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SUMMARY

Interleukin-17 (IL-17) induces pathology in autoimmunity and infections; therefore, constraint of this pathway is an essential component of its regulation. We demonstrate that the signaling intermediate MCPIP1 (also termed Regnase-1, encoded by *Zc3h12a*) is a feedback inhibitor of IL-17 receptor signal transduction. MCPIP1 knockdown enhanced IL-17-mediated signaling, requiring MCPIP1's endoribonuclease but not deubiquitinase domain. MCPIP1 haploinsufficient mice showed enhanced resistance to disseminated *Candida albicans* infection, which was reversed in an *Il17ra*$^{-/-}$ background. Conversely, IL-17-dependent pathology in *Zc3h12a*$^{+/-}$ mice was exacerbated in both EAE and pulmonary inflammation. MCPIP1 degraded *Il6* mRNA directly but only modestly downregulated the IL-6 promoter. However, MCPIP1 strongly inhibited the *Lcn2* promoter by regulating the mRNA stability of *Nfkbiz*, encoding the IκBζ transcription factor. Unexpectedly, MCPIP1 degraded *Il17ra* and *Il17rc* mRNA, independently of the 3′ UTR. The cumulative impact of MCPIP1 on IL-6, IκBζ, and possibly IL-17R subunits results in a biologically relevant inhibition of IL-17 signaling.

INTRODUCTION

IL-17 (IL-17A) is the signature cytokine of Th17 cells and is also produced by innate immune cells (Cua and Tato, 2010). IL-17-deficient individuals experience recurrent fungal and bacterial infections caused by *Candida* and *Staphylococcal* species (Milner and Holland, 2013). Conversely, IL-17 is a major driver of autoimmunity, and antibodies targeting IL-17 or its receptor are showing promise in treating psoriasis and other disorders (Patel et al., 2013).

IL-17 and its receptor, a heterodimer of IL-17RA and IL-17RC, are all members of a distinct class of cytokines (Gaffen et al.,

2014). Both subunits contain a motif known as SEFIR (SEF and IL-17R) domain, which engages the only known SEFIR-containing adaptor, Act1. Act1 recruits and ubiquitinates TRAF6, activating downstream NF-κB, CCAAT/Enhancer Binding Protein (C/EBP), and Mitogen Activated Protein Kinase (MAPK) pathways. IL-17-induced targets include cytokines (IL-6, G-CSF), antimicrobial peptides (S100A8/9, lipocalin-2, β-defensins), transcription factors (C/EBPδ, IκBζ), and neutrophil-recruiting chemokines (CXCL1, CXCL2, CXCL5). In addition to stimulating transcription, IL-17 controls stabilization of mRNA transcripts through RNA binding proteins such as SF2 and HuR (Herjan et al., 2013; Sun et al., 2011). Thus, IL-17 signaling upregulates a program of gene expression that controls infection.

Dysregulated inflammation causes collateral tissue damage, ultimately resulting in lymphoproliferative disorders or autoimmunity. This process is well studied for TNF and IL-1 signaling but is poorly defined for IL-17. Recently, several checkpoints have been identified that restrict IL-17 activity. The deubiquitinases (DUBs) A20 and USP25 negatively regulate IL-17-induced NF-κB and MAPK activation by removing ubiquitin modifications on TRAF6 (Garg et al., 2013; Zhong et al., 2012). Additionally, IL-17 suppresses expression of miR-23b, a microRNA that limits NF-κB activation by targeting TAB2, TAB3, and IKKα (Song and Qian, 2013; Zhu et al., 2012). IL-17 also induces phosphorylation of C/EBPβ, a transcription factor that inhibits IL-17-dependent gene expression (Shen et al., 2009).

In an effort to discover new regulators of IL-17 signal transduction, we evaluated archival microarray data for potential IL-17-dependent signaling modulators. *Zc3h12a* (encoding MCPIP1; also known as Regnase-1) was identified in a screen of IL-17RA-dependent genes involved in antifungal immunity (Conti et al., 2009). This gene is also induced in fibroblasts or macrophages treated with IL-17 (Dhamija et al., 2013; Sønder et al., 2011). MCPIP1 is expressed at low levels in most cell types but is upregulated by inflammatory stimuli such as MCP-1, IL-1β, and TLR ligands (Jura et al., 2012). MCPIP1 is an endoribonuclease (RNase) that inhibits TLR signaling by degrading mRNA transcripts such as *Il6* through a 3′ UTR stem-loop motif (Matsushita et al., 2009; Mino et al., 2015). IL-6 is a downstream gene target of IL-17 signaling and contributes to many
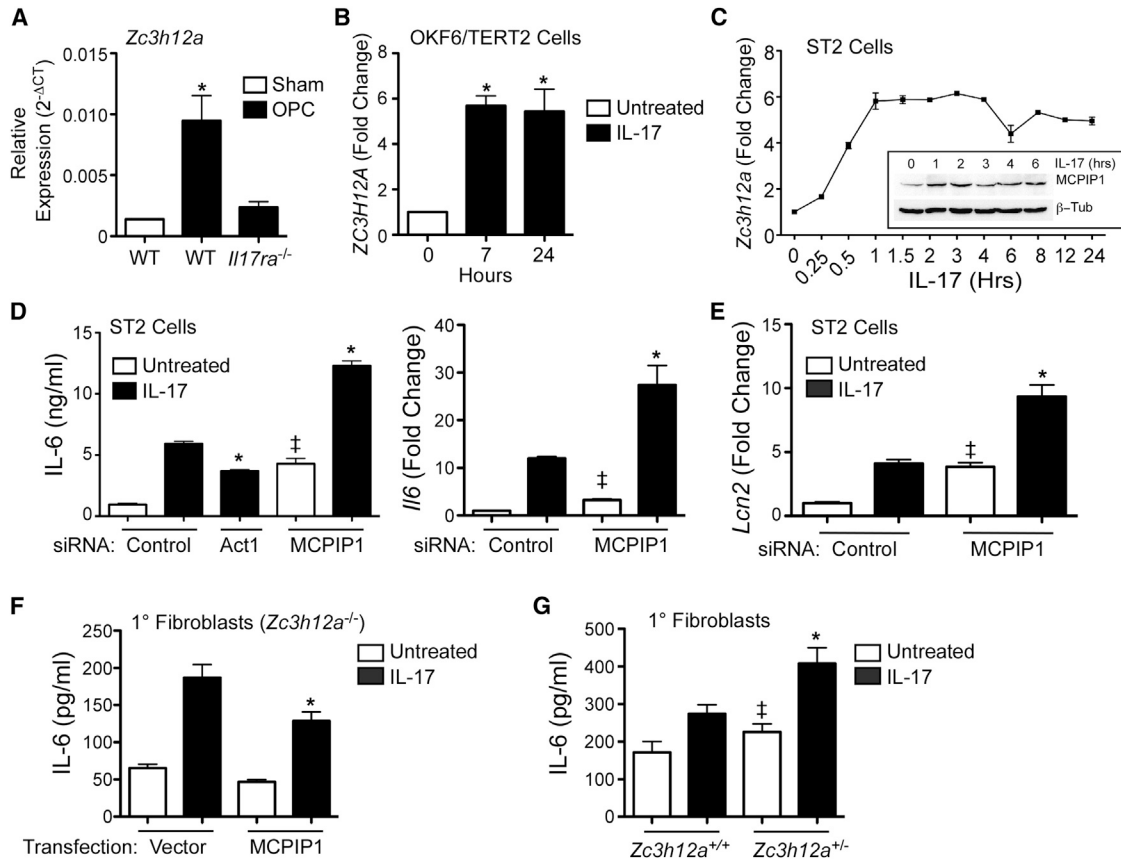
Figure 1. MCPIP1 Is a Feedback Inhibitor of IL-17 Signaling

(A) RNA from WT or $Il17ra^{-/-}$ tongues (n = 3) were collected 48 hr after oral infection with PBS (sham) or *C. albicans*. Expression of *Zc3h12a* was assessed by qPCR and normalized to *Gapdh*. *p < 0.05 versus sham.

(B) OKF6/TERT2 oral keratinocytes were treated ± IL-17 and mRNA analyzed for *ZC3H12A* by qPCR. Data presented as fold change versus unstimulated (0 hr); *p < 0.05 versus untreated.

(C) ST2 cells were incubated ± IL-17 and mRNA was analyzed by qPCR, normalized to *Gapdh*. Inset: Lysates from ST2 cells treated ± IL-17 were immunoblotted for MCPIP1 or β-tubulin. Data presented as fold change versus unstimulated.

(D and E) ST2 cells were transfected with siRNAs against Act1, MCPIP1, or a scrambled control. Cells were treated ± IL-17 for 3 hr. Supernatants were assessed by ELISA (left). *Il6* and *Lcn2* were assessed by qPCR. *p < 0.05 versus IL-17-treated siRNA control; ‡p < 0.05 versus untreated. Data presented as fold change relative to untreated siRNA control.

(F) $Zc3h12a^{-/-}$ fibroblasts were transfected with an empty vector or a plasmid encoding murine MCPIP1. Cells were treated with IL-17 for 4 hr, and IL-6 assessed by ELISA. *p < 0.05 versus IL-17-treated controls.

(G) Primary fibroblasts from $Zc3h12a^{+/-}$ or WT littermates were treated with IL-17 for 24 hr and IL-6 assessed by ELISA.*p < 0.05 versus IL-17-treated controls. ‡p < 0.05 versus untreated.

Data presented as mean ± SEM throughout. All experiments were performed a minimum of twice.

IL-17-mediated inflammatory events (Camporeale and Poli, 2012). In addition to its RNase functions, MCPIP1 exhibits DUB activity that blocks TLR-activation of TRAFs (Liang et al., 2010; Niu et al., 2013). In vivo, MCPIP1 is a negative regulator of TLR and TCR signaling (Liang et al., 2010; Uehata et al., 2013) and limits Th17 cell differentiation (Jeltsch et al., 2014).

We show that MCPIP1 is a direct inhibitor of IL-17-mediated signal transduction. MCPIP1 deficiency enhanced IL-17-dependent resistance to *Candida albicans* infection and also increased pathology in experimental autoimmune encephalomyelitis (EAE). These effects were associated with suppressed expression of IL-6, Lipocalin 2, IκBζ, and other IL-17 target genes. In addition to its capacity to degrade mRNA, we found that MCPIP1 regulates certain IL-17-dependent promoters through control of

IκBζ. Lastly, MCPIP1 can induce decay of mRNA transcripts encoding inflammatory receptors, including IL-17R subunits.

RESULTS

MCPIP1 Is a Feedback Inhibitor of IL-17 Signaling

Mice and humans lacking the IL-17 receptor are susceptible to *C. albicans* infection (Milner and Holland, 2013). We observed that *Zc3h12a* was rapidly induced after oropharyngeal candidiasis (OPC) in WT but not $Il17ra^{-/-}$ mice (Figure 1A; Conti et al., 2009). Consistently, *ZC3H12A* was induced by IL-17 in human oral keratinocytes (Figure 1B; Dickson et al., 2000). In ST2 cells, a murine stromal cell line, expression of *Zc3h12a* was enhanced within 30 min of IL-17 stimulation and remained ~5- to 6-fold

elevated for at least 24 hr. MCPIP1 protein expression was increased with similar kinetics (Figure 1C).

To determine whether MCPIP1 impacts IL-17 signaling, ST2 cells were transfected with siRNAs targeting MCPIP1, Act1, or a scrambled control. Efficiency of knockdown was 50%–70% (Figures S1A and S1B). Cells were treated with IL-17 for 3 hr, and IL-6 was assessed in conditioned media. As expected, Act1 knockdown inhibited IL-17-induced production of IL-6 (Figure 1D). In contrast, knockdown of MCPIP1 increased IL-17-dependent IL-6 secretion and *Il6* mRNA (Figure 1D). MCPIP1 knockdown also increased basal expression of IL-6, revealing a role in regulating tonic cytokine expression. Because *Il6* is a known target of MCPIP1 (Matsushita et al., 2009), we evaluated additional IL-17-dependent genes to assess specificity. Similarly, *Csf3* (G-CSF) was enhanced upon MCPIP1 knockdown (Figure S1A). Unlike *Il6*, IL-17 induction of *Lcn2* (lipocalin 2) is not controlled at the level of mRNA stability (Shen et al., 2006). Nonetheless, MCPIP1 knockdown increased IL-17-dependent expression of *Lcn2* (Figure 1E). To rule out off-target effects of siRNA, *Zc3h12a*$^{-/-}$ fibroblasts were transfected with a plasmid containing MCPIP1 or a control vector. Reconstitution of *Zc3h12a*$^{-/-}$ cells led to reduced IL-6 expression in response to IL-17 (Figure 1F). Consistently, fibroblasts from *Zc3h12a*$^{+/-}$ and *Zc3h12a*$^{-/-}$ mice showed enhanced induction of IL-6 in response to IL-17 (Figures 1G, S1C, and S1D). Thus, MCPIP1 is a feedback inhibitor of IL-17 signaling in non-hematopoietic cells.

MCPIP1 Inhibits IL-17 Signaling in Pulmonary Inflammation

Zc3h12a$^{-/-}$ mice exhibit severe inflammation due to unrestrained TLR signaling from intestinal microbiota (Liang et al., 2010; Matsushita et al., 2009; Miao et al., 2013). Because these mice survive only ~6–8 weeks, they were unsuitable for experimentation. However, *Zc3h12a*$^{+/-}$ mice are healthy, fertile, and exhibit normal lifespans. There was no detectable baseline inflammation in visceral organs (lungs or kidneys) of unmanipulated *Zc3h12a*$^{+/-}$ mice determined by expression of inflammatory cytokines (Figure S2), though they showed mild inflammation in gut (data not shown). Moreover, fibroblasts from *Zc3h12a*$^{+/-}$ mice showed reduced expression of MCPIP1 upon treatment with inflammatory stimuli such as LPS and IL-17, whereas baseline levels were similar to *Zc3h12a*$^{+/+}$ (Figure 2A). These findings raised the possibility that *Zc3h12a*$^{+/-}$ mice might have impaired control of inflammation in settings where MCPIP1 is normally induced (namely, IL-17 signaling).

To determine whether MCPIP1 haploinsufficiency led to enhanced IL-17 activity in vivo, *Zc3h12a*$^{+/-}$ or *Zc3h12a*$^{+/+}$ littermates were challenged intranasally with IL-17 to induce pulmonary inflammation. After 24 hr, bronchoalveolar lavage fluid (BALF) was evaluated for cellular influx and IL-17-dependent inflammation. The number and percentage of Gr1$^+$F4/80$^-$ infiltrating neutrophils in *Zc3h12a*$^{+/-}$ BALF were elevated compared to *Zc3h12a*$^{+/+}$ (Figures 2B and 2C). Histology confirmed enhanced inflammation in *Zc3h12a*$^{+/-}$ lungs (Figure 2D). Consistent with elevated neutrophil infiltration, there was also increased amounts of CXCL5 in the BALF, although CXCL1 concentrations were unchanged at this time point (Figure 2E). Notably, no T cells were recruited to lung in this setting (J.K.K., unpublished data).

These results demonstrate that *Zc3h12a*$^{+/-}$ mice exhibit an elevated capacity for IL-17 responsiveness.

MCPIP1 Deficiency Enhances IL-17-Mediated Resistance to Fungal Infection

Because IL-17 drives immunity to *C. albicans* (Huang et al., 2004; Saijo et al., 2010), we postulated that elevated IL-17 signaling in *Zc3h12a*$^{+/-}$ mice would improve fungal clearance. Accordingly, mice were inoculated i.v. with *C. albicans*, and kidney fungal loads were assessed after 2 days. *Zc3h12a*$^{+/-}$ mice exhibited a 2-log lower renal fungal load than *Zc3h12a*$^{+/+}$ mice (Figure 3A) and markedly prolonged survival (Figure 3B). PAS staining confirmed the presence of invasive *Candida* hyphae in *Zc3h12a*$^{+/+}$ but not *Zc3h12a*$^{+/-}$ kidneys (Figure 3C). To verify that the reduced fungal load in *Zc3h12a*$^{+/-}$ mice was IL-17 dependent, *Zc3h12a*$^{+/-}$ mice were crossed to *Il17ra*$^{-/-}$ mice and subjected to candidiasis. As previously shown (Huang et al., 2004), *Il17ra*$^{-/-}$ mice exhibited ~1-log higher fungal burden than WT (Figure 3A). Strikingly, *Zc3h12a*$^{+/-}$*Il17ra*$^{-/-}$ mice were unable to control infection, with a renal fungal load ~2.5-log greater than *Zc3h12a*$^{+/-}$ and indistinguishable from *Il17ra*$^{-/-}$ mice (Figure 3A). *Zc3h12a*$^{+/-}$*Il17ra*$^{-/-}$ mice also exhibited decreased survival in response to infection (Figure S2C). Therefore, the reduced *Candida* susceptibility in *Zc3h12a*$^{+/-}$ mice is due to enhanced IL-17 signaling.

IL-6 is one of the best characterized IL-17 target genes and contributes to immunity to candidiasis (Basu et al., 2008). Accordingly, if the entire protective effect of MCPIP1 were due to IL-6, IL-6 neutralization would be expected to reverse disease susceptibility. To test this hypothesis, *Zc3h12a*$^{+/+}$ or *Zc3h12a*$^{+/-}$ mice were administered neutralizing Abs against IL-6R (Wu et al., 2013) and infected with *C. albicans*. As expected, blocking IL-6 increased fungal loads in both *Zc3h12a*$^{+/+}$ and *Zc3h12a*$^{+/-}$ mice (Figure 3D). However, anti-IL-6R Abs did not fully reverse the protection in *Zc3h12a*$^{+/-}$ animals, suggesting that IL-6 is not the sole IL-17-dependent mediator of disease protection. This is consistent with findings that no change in tonic inflammation occurs in *Zc3h12a*$^{-/-}$*Il6*$^{-/-}$ mice (Uehata et al., 2013).

MCPIP1 Deficiency Enhances EAE

IL-17 drives pathology in EAE, so we predicted that *Zc3h12a*$^{+/-}$ mice would show enhanced CNS inflammation due to unrestrained IL-17 signaling. Mice were immunized with myelin oligodendrocyte (MOG) in CFA and administered one dose of pertussis toxin to cause mild clinical EAE. *Il17ra*$^{-/-}$ mice were resistant to EAE, whereas *Zc3h12a*$^{+/-}$ mice showed dramatically increased clinical scores, with earlier disease onset and higher incidence (Figure 4A). These events were IL-17 dependent, as indicated by the fact that *Zc3h12a*$^{+/-}$*Il17ra*$^{-/-}$ mice exhibited reduced EAE symptoms compared to *Zc3h12a*$^{+/-}$ mice. Spinal cord showed enhanced expression of IL-17 target genes known to contribute to EAE, including *Il6*, *Cxcl1*, and *Csf2* (Figure 4B). Thus, MCPIP1 constrains autoimmune pathology driven by IL-17.

MCPIP1 Regulates *Nfkbiz* mRNA Stability

IL-17 upregulates IL-6 expression by control of mRNA stability and by activating its proximal promoter (Ruddy et al., 2004). To determine whether MCPIP1 impacts the IL-6 promoter, ST2 cells
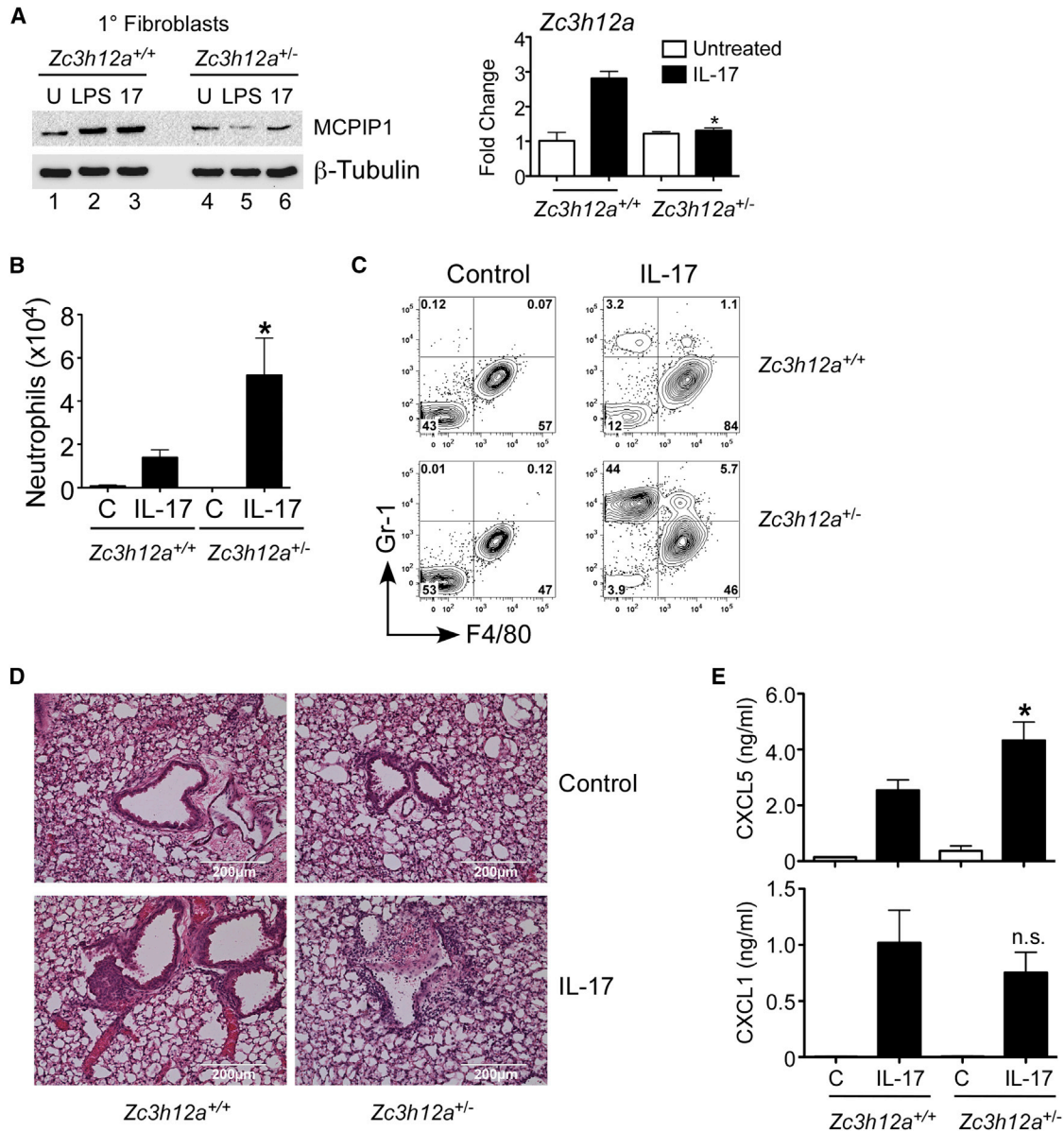
Figure 2. MCPIP1 Inhibits IL-17-Mediated Pulmonary Inflammation in *Zc3h12a*^{+/−} Mice

(A) Fibroblasts from *Zc3h12a*^{+/−} mice or WT littermates were treated with LPS or IL-17 for 4 hr and MCPIP1 and β-tubulin assessed by immunoblotting (left) or qPCR (right). Data expressed as fold-change versus untreated WT. *$p < 0.05$ versus IL-17-treated WT sample.

(B and C) WT or *Zc3h12a*^{+/−} mice (n = 5) were treated intranasally with 300 ng recombinant IL-17. 24 hr later, BALF was stained for Gr-1 and F4/80 and quantified by flow cytometry. C = control unchallenged. *$p < 0.05$ compared to IL-17-treated WT mice.

(D) H&E-stained lung sections from the indicated mice are shown. Scale bars represent 200 μM.

(E) WT or *Zc3h12a*^{+/−} mice (n = 2 for control, n = 5 for IL-17-treated) were treated intranasally with 500 ng IL-17. After 8 hr, levels of CXCL5 and CXCL1 in BALF were assessed by ELISA. *$p < 0.05$ versus IL-17-treated WT mice. n.s., not significant.

Data are presented as mean ± SEM throughout. All experiments were performed a minimum of twice.

were co-transfected with MCPIP1 and a luciferase reporter driven by the IL-6 proximal promoter (Eickelberg et al., 1999). MCPIP1 modestly suppressed IL-17-mediated activation of the IL-6 promoter (~15% reduction, Figure 5A), indicating that the enhanced expression of IL-6 seen after MCPIP1 knockdown is due mainly to *Il6* transcript stabilization.

IL-17 also activates the *Lcn2* promoter but does not promote *Lcn2* mRNA half-life (Shen et al., 2006). In contrast to the IL-6 promoter, MCPIP1 strongly suppressed the *Lcn2* promoter (~85%, Figure 5A). The baseline activities of the IL-6 and Lcn2 promoters were also suppressed, consistent with the inhibitory effect of MCPIP1 on tonic expression of these genes (Figure 1). Because MCPIP1 is not a transcription factor, it was unlikely that its activity on the Lcn2 promoter was direct. Therefore, we evaluated the impact of MCPIP1 on signaling intermediates downstream of IL-17. MCPIP1 knockdown enhanced
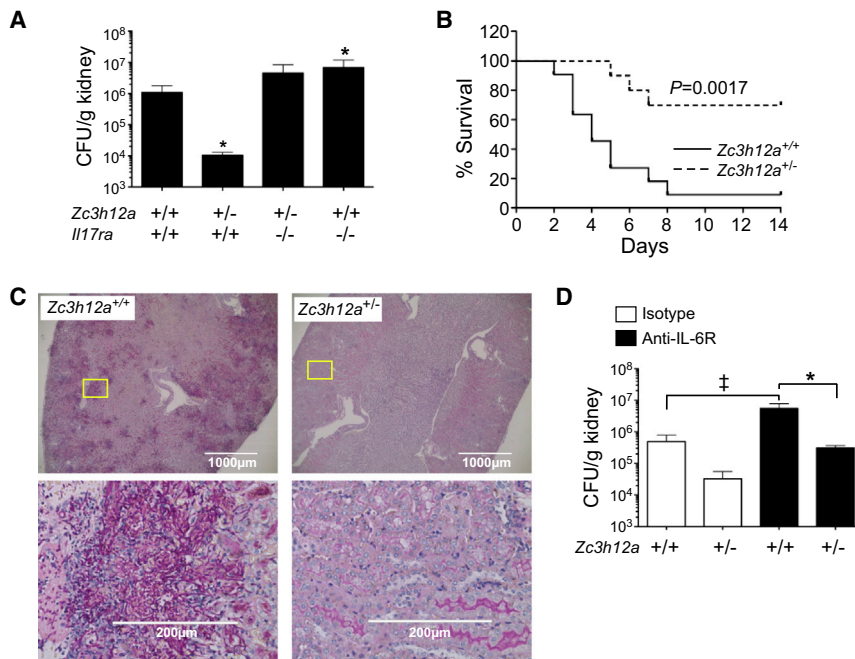
Figure 3. MCPIP1 Restrains IL-17-Dependent Responses to *Candida albicans* Infection

(A) *Zc3h12a*$^{+/-}$ mice crossed to *Il17ra*$^{-/-}$ mice (n = 5–8) and littermates were subjected to candidiasis by i.v. injection of *C. albicans*. After 2 days, fungal burdens in kidney were assessed by plating and colony enumeration. *p < 0.05 versus WT. Data pooled from two independent experiments.

(B) Survival curve of WT (solid line, n = 11) and *Zc3h12a*$^{+/-}$ (dashed line, n = 10) mice after candidiasis. Data are pooled from two independent experiments.

(C) Periodic acid Schiff (PAS)-stained kidney sections. Yellow boxes indicate location of higher magnification images. Scale bars represent 1,000 μM (top) or 200 μM (bottom).

(D) WT or *Zc3h12a*$^{+/-}$ mice (n = 4–6) were infected i.v. with *C. albicans* after administration of α-IL-6R or isotype Abs. Fungal loads in kidney were assessed 2 days after infection. *p < 0.05 versus α-IL-6R-treated WT mice. ‡p < 0.05 versus isotype-treated WT mice.

Data presented as mean ± SEM throughout. All experiments were performed a minimum of twice.

expression of *Nfkbiz* (encoding IκBζ) and *Rel* (encoding c-Rel) at baseline and upon IL-17 stimulation (Figures 5B and S3). There was no effect on other NF-κB components, C/EBPβ, C/EBPδ, TAB2, or TAB3, consistent with the known activity of MCPIP1 in T cells (Figure S3; Uehata et al., 2013). We postulated that inhibition of IκBζ expression by MCPIP1 might explain its potent effect on Lcn2 promoter activation (Figure 5A; Karlsen et al., 2010). Indeed, co-expression of MCPIP1 with IκBζ in HEK293T cells led to marked degradation of IκBζ (Figure 5C, lane 2), which required its 3′ UTR (Figure 5C, lane 4). To determine whether IκBζ in turn regulated *Lcn2*, ST2 cells were treated with siRNA against IκBζ or MCPIP1 and treated with IL-17 for 3 hr. Knockdown of IκBζ abrogated induction of *Lcn2* (Figure 5D), as well as other IL-17 target genes, including *Cxcl1*, *Cxcl5*, and *Ccl20* (Figure S4A). Accordingly, MCPIP1 impairs activation of some IL-17-dependent promoters through degradation of *Nfkbiz*.

MCPIP1 Degrades Inflammatory Receptor mRNA

USP25 and A20 temper IL-17 signaling by deubiquitinating TRAF6 (Garg et al., 2013; Zhong et al., 2012), and MCPIP1 also possesses deubiquitinase (DUB) activity (Liang et al., 2010). To determine whether MCPIP1 acts redundantly with USP25 or A20, ST2 cells were transfected with siRNAs targeting MCPIP1, A20, or USP25. Knockdown of MCPIP1 in combination with A20 or USP25 led to increased induction of IL-6 compared to knockdown of each inhibitor alone (Figures 6A and S5E), indicating that they act by nonredundant mechanisms. Recently the RNA binding proteins Roquin-1 and Roquin-2 were shown to regulate *Il6* (Jeltsch et al., 2014). As with A20 and USP25, we saw an additive effect of knocking down both MCPIP1 and Roquins on *Il6* and *Lcn2* mRNA, although not detectably for IL-6 protein, suggesting that they are not fully redundant in the IL-17 pathway (Figure S4B).

A20 and TRAF3 bind directly to the IL-17RA cytoplasmic tail to limit signaling (Garg et al., 2013; Zhu et al., 2010). Consequently, we asked whether MCPIP1 binds to the IL-17R. Myc-tagged murine (m)IL-17RA, mIL-17RC, or Act1 were expressed in HEK293T cells with Flag-MCPIP1. Lysates were immunoprecipitated with anti-Myc Abs and immunoblotted for Flag and Myc. Unexpectedly, co-expression of MCPIP1 with IL-17RA or IL-17RC resulted in a strong, dose-dependent decrease in receptor expression (Figures 6B, lanes 6–7, and 6C), making it impossible to determine whether MCPIP1 binds these subunits. In contrast, there was no effect of MCPIP1 on expression of TRAF6, Act1, or signaling-defective Act1 mutants (Figures 6B, lane 8, and S5A and S5C). Transfection of MCPIP1 with TLR4 and TNFR2 also diminished their expression (Figure S5B). To assess whether the decrease in IL-17RA impacted its expression at the cell surface, mIL-17RA and MCPIP1 were transfected into HEK293T cells and mIL-17RA expression was evaluated by flow cytometry. Co-expression with MCPIP1 but not TRAF6 or A20 led to reduced surface expression of mIL-17RA. An RNase-deficient mutant of MCPIP1 (Liang et al., 2010) did not alter IL-17RA surface expression (Figure 6D). To determine whether MCPIP1 downregulates IL-17RA by degrading its mRNA, MCPIP1, IL-17R subunits, or Act1 were co-transfected in HEK293T cells. Indeed, there was markedly reduced expression of *Il17ra* and *Il17rc* mRNA but not *Traf3ip2* (Act1) in the presence of MCPIP1 (Figure 6E).

The observation that MCPIP1 degrades IL-17R transcripts was surprising, because they lack prototypical destabilizing AU-rich elements (AREs) in the 3′ UTR. We therefore mapped the target sequence needed for degradation with a series of IL-17RA mutants (Figures S6A–S6C). A mIL-17R construct containing the first 775 nucleotides (258 amino acids) was subject to MCPIP1 degradation, whereas a mutation with just the first 600 nucleotides was not (Figure 6F). Consistently, a mIL-17RA
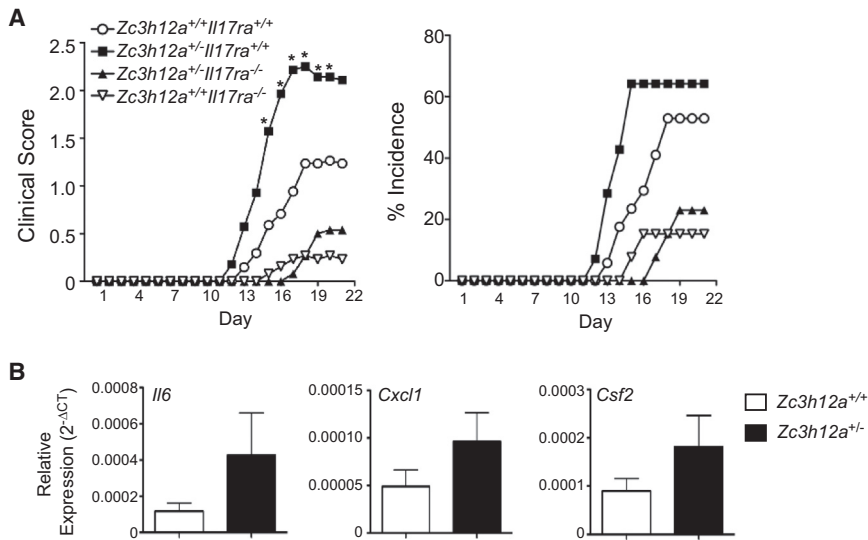
Figure 4. MCPIP1 Limits IL-17-Dependent Autoimmune CNS Pathology

(A) The indicated mice (n = 13–17) were subjected to EAE and clinical scores assessed daily (left). The percentage of mice exhibiting EAE symptoms is indicated (right). Data are pooled from two experiments. Data are presented as mean clinical score of all mice. *p < 0.05 by ANOVA and Student's t test.

(B) Gene expression in spinal cords was measured by qPCR normalized to *Gapdh*.

Data presented as mean ± SEM throughout.

construct with an internal deletion spanning this sequence (IL-17RAΔ200–258) was largely, though not completely, resistant to degradation, observed at both mRNA and protein levels (Figure 6F). Thus, MCPIP1 degrades *Il17ra* through a motif located in its 5′ coding region.

To show that MCPIP1 degraded *Il17ra* mRNA directly, His-tagged MCPIP1 was purified from transfected HEK293T cells (Figure S6D). RNAs encoding the *Il6*-3′ UTR or *Il17ra* (bases 1–775, as in Figure 6F) were generated in vitro, co-incubated for 1 hr with purified MCPIP1, and visualized on denaturing agarose gels (Figure 6G). The *Il6*-3′UTR and *Il17ra* transcripts were efficiently degraded by MCPIP1, but *Traf3ip2* mRNA (Act1) was resistant to degradation (Figure S6E). There was reduced migration of all residual transcripts in lanes where purified MCPIP1 was added, suggesting that MCPIP1 binds directly to mRNA. Because *Traf3ip2* mRNA also showed slower migration, binding of MCPIP1 to a putative target might be insufficient to drive substrate degradation. To determine whether a decrease in *Il17ra* mRNA occurred in vivo, *Il17ra* was assessed in spinal cords of mice subjected to EAE, a setting where dynamic regulation of IL-17RA has been reported (Liu et al., 2014a). Expression of *Il17ra* mRNA was indeed enhanced, albeit modestly, in *Zc3h12a*^{+/−} spinal cord homogenates compared to *Zc3h12a*^{+/+} (Figure S6F). We confirmed by flow cytometry that IL-17RA surface expression was induced in microglia during EAE, which had not been previously shown. However, there was no detectable elevation of IL-17RA in *Zc3h12a*^{+/−} compared to *Zc3h12a*^{+/+} microglia (Figure S6G). Collectively, these data show that MCPIP1 has the capacity to degrade *Il17ra* mRNA through its 5′ coding sequence. Although *IL17ra* mRNA was elevated in *Zc3h12a*^{+/−} spinal cords during EAE, there was no apparent impact on IL-17RA surface expression, indicating that this phenomenon probably does not account for the enhanced susceptibility to EAE in *Zc3h12a*^{+/−} animals.

We next sought to delineate the MCPIP1 domain required to mediate mRNA degradation (Figure 7A). Mutants of human MCPIP1 in the RNase domain (D225/226A and D141N), a zinc finger (ZnF) domain deletion (ΔZF), or a point mutation within the ZnF (C306R) were co-expressed with mIL-17RA in HEK293T

cells, and IL-17RA was assessed by immunoblotting. As expected, ZnF mutants of MCPIP1 failed to induce IL-17RA degradation (Figure 7B, lanes 1, 3, 5). The ZnF is required for both RNase and DUB activity (Liang et al., 2010), but a ΔDUB domain mutant (Kapoor et al., 2015) degraded IL-17RA similarly to WT (Figure 7C, lane 3). Similarly, degradation of IκBζ occurred independently of the DUB domain (Figure 7D). *Zc3h12a*^{−/−} fibroblasts reconstituted with WT but not ZnF-deficient MCPIP1 (C306R) showed impaired IL-17-induced IL-6 production (Figure 7E). These data confirm that the RNase activity of MCPIP1 regulates IL-17-dependent signaling. Because ZnF-deficient MCPIP1 mutants did not cause IL-17RA degradation, we re-visited the question of whether MCPIP1 and IL-17RA proteins interact directly by co-transfecting IL-17RA with MCPIP1.ΔZF and MCPIP1.D225/226A. However, there was no apparent association of MCPIP1 with IL-17RA (Figure S5D). Thus, unlike A20 or TRAF3 (Garg et al., 2013; Zhu et al., 2010), MCPIP1 does not directly engage the IL-17R, but rather targets downstream mRNA transcripts for degradation.

DISCUSSION

IL-17 is vital for controlling fungal and bacterial infections, both in mice and humans (Milner and Holland, 2013). IL-17 exerts its host-protective activity by upregulating antimicrobial peptides, cytokines, and chemokines as well as transcription factors that regulate those genes (Onishi and Gaffen, 2010). Conversely, IL-17 inhibition limits collateral damage stemming from IL-17-induced inflammation, which is key to controlling autoimmunity. This concept is being exploited clinically with biologics targeting IL-17, the IL-17R, or Th17 cells (Gaffen et al., 2014).

To identify new signaling molecules that regulate the IL-17 pathway, we mined published data comparing IL-17R-dependent gene expression in the context of *Candida* infection (Conti et al., 2009), thereby identifying *Zc3h12a* as an IL-17 target gene. MCPIP1 was originally discovered as a target of MCP-1 and is upregulated by many inflammatory stimuli (Dhamija et al., 2013; Sønder et al., 2011). IL-17 regulates *Zc3h12a* through Act1-mediated activation of NF-κB and IKKγ as well as through mRNA stabilization (Somma et al., 2015; Sønder et al., 2011). MCPIP1 belongs to a family of CCCH zinc finger proteins that exhibit RNase activity against inflammatory and viral mRNA transcripts (Uehata and Akira, 2013; Xu et al.,
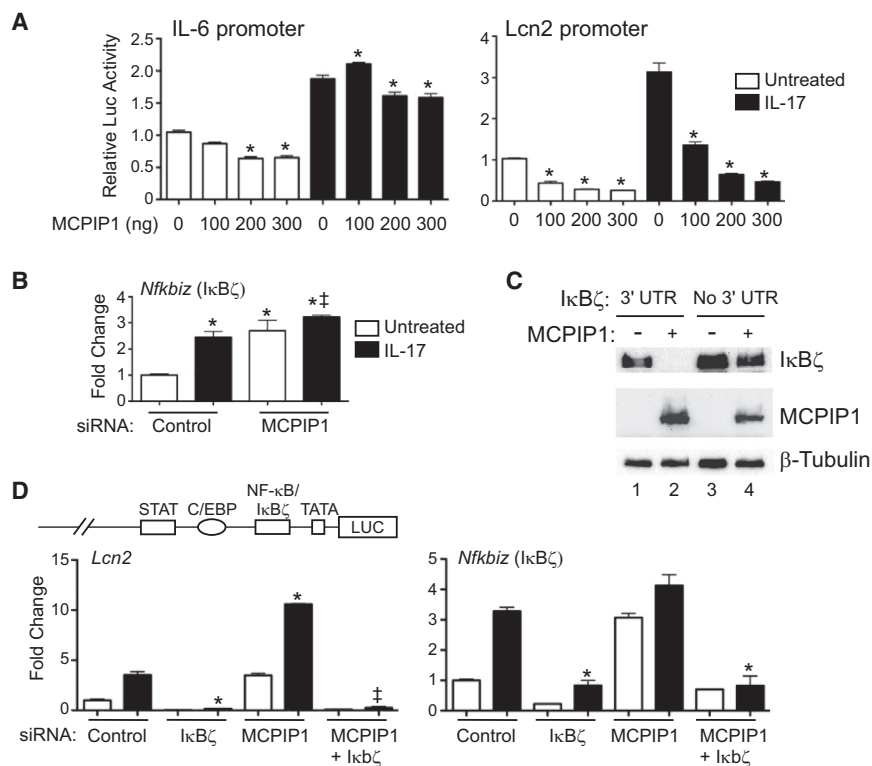
Figure 5. MCPIP1 Differentially Regulates IL-17 Target Promoters and Transcription Factors

(A) ST2 cells were transfected with varying doses of MCPIP1 plasmid (0–300 ng) together with Luc reporters driven by the IL-6 or Lcn2 promoters. Cells were treated ± IL-17 for 8 hr and Luc activity assessed in triplicate. Data presented relative to the unstimulated control without MCPIP1. *p < 0.05 versus unstimulated sample of the corresponding condition.

(B) ST2 cells were transfected with siRNAs against MCPIP1, stimulated ± IL-17 for 3 hr, and analyzed for expression of the indicated genes by qPCR. *p < 0.05 versus unstimulated. ‡p < 0.05 versus IL-17-treated control siRNA.

(C) HEK293T cells were transfected with MCPIP1 ± constructs expressing IκBζ with or without its 3′ UTR. Whole cell lysates were analyzed by immunoblotting for IκBζ (top), MCPIP1 (middle), or β-tubulin (bottom).

(D) Top: Murine Lcn2 promoter construct. Bottom: ST2 cells were transfected with siRNAs targeting IκBζ, MCPIP1, or a scrambled control, treated ± IL-17 for 3 hr, and the indicated genes evaluated by qPCR. Data expressed as fold-change relative to the untreated control siRNA. *p < 0.05 versus control siRNA sample treated with IL-17. ‡p < 0.05 versus MCPIP1 siRNA treated with IL-17.

Data presented as mean ± SEM throughout. Experiments were performed a minimum of twice.

2012). Additionally, MCPIP1 inhibits TLR4 signaling by deubiquitinating TRAFs (Liang et al., 2010). Because IL-17 shares overlapping activation mechanisms and target genes with TLRs, we postulated that MCPIP1 might inhibit the IL-17 pathway. RNA silencing and reconstitution of $Zc3h12a^{-/-}$ cells confirmed this hypothesis, which was further supported in a variety of IL-17-dependent in vivo models. MCPIP1 thus joins the ranks of A20 and miR-23b as negative feedback regulators of IL-17 signal transduction.

In contrast to its DUB functions in blocking TLR activation, only the RNase activity of MCPIP1 seems to be critical in blocking IL-17 signals. Control of mRNA half-life is an essential but often overlooked facet of immune homeostasis (Hamilton et al., 2010). IL-17 promotes stability of many inflammatory gene transcripts, best studied for *Cxcl1* (Datta et al., 2010). IL-17-induced stabilization of *Cxcl1* occurs through Ikki-induced phosphorylation of Act1. In turn, phosphorylated Act1 binds TRAF2 and TRAF5, which sequester the RNA binding protein SF2 to allow binding of the mRNA-stabilizing factor HuR (Bulek et al., 2011; Herjan et al., 2013; Sun et al., 2011). IL-17, in cooperation with TNFα, promotes *Il6* mRNA stability, mediated in part through MAPK (Shimada et al., 2002; Tokuda et al., 2004). Here we show that MCPIP1, via its RNase activity, controls expression of a variety of IL-17-induced genes. Although some of this regulation might be through direct action on target transcripts (e.g., *Il6*), certain IL-17 target genes such as *Lcn2* are regulated through their promoters, which we show occurs indirectly via *Nfkbiz*. It is possible that microRNAs or regulation of other signaling intermediates such as c-Rel might additionally contribute to the activity of MCPIP1 in the IL-17 cascade, similar to recent findings for regulation of A20 by miR-873 (Liu et al., 2014b). However, MCPIP1 inhibits DICER, affecting miR biogenesis nonspecifically (Suzuki et al., 2011). miR-23b inhibits IL-17 signaling by degrading TAB2/3 and IKKα (Zhu et al., 2012); therefore, inhibition of DICER would be expected to decrease miR-23b, leading to increased expression of NF-κB-dependent genes such as *Il6* and *Lcn2*. Because we see the opposite effect of MCPIP1 on these genes, it is unlikely that the miR-23b pathway is a target of MCPIP1.

MCPIP1 regulates tonic inflammation and is vital for maintaining immune homeostasis in vivo. Consequently, $Zc3h12a^{-/-}$ mice suffer from fatal multi-organ inflammation (Liang et al., 2010; Matsushita et al., 2009). This effect is attributed to TLR signaling from gut microbiota, because antibiotics extend $Zc3h12a^{-/-}$ lifespan (Huang et al., 2013; Liang et al., 2010, 2011; Miao et al., 2013). We generated $Zc3h12a^{-/-}Il17ra^{-/-}$ double knockout mice that were not rescued from systemic inflammation or early death, indicating that IL-17 is not a major driver of spontaneous hyperinflammation (data not shown). In contrast, $Zc3h12a^{+/-}$ mice did not exhibit early death or systemic inflammation in visceral organs. $Zc3h12a^{+/-}$ mice showed reduced expression of MCPIP1 after stimulation with LPS or IL-17; thus, the impact of MCPIP1 haploinsufficiency apparently manifests mainly in inflammatory conditions.

Inflammation controlled by MCPIP1 is also linked to enhanced T cell activation and subsequent impairment of Th17 cell development (Gewies et al., 2014; Jeltsch et al., 2014; Uehata et al., 2013). In T cells, inhibitory activity is constitutive, as MCPIP1 is cleaved by MALT1 upon TCR activation, relieving its checkpoint activity (Jeltsch et al., 2014; Mino et al., 2015). However,
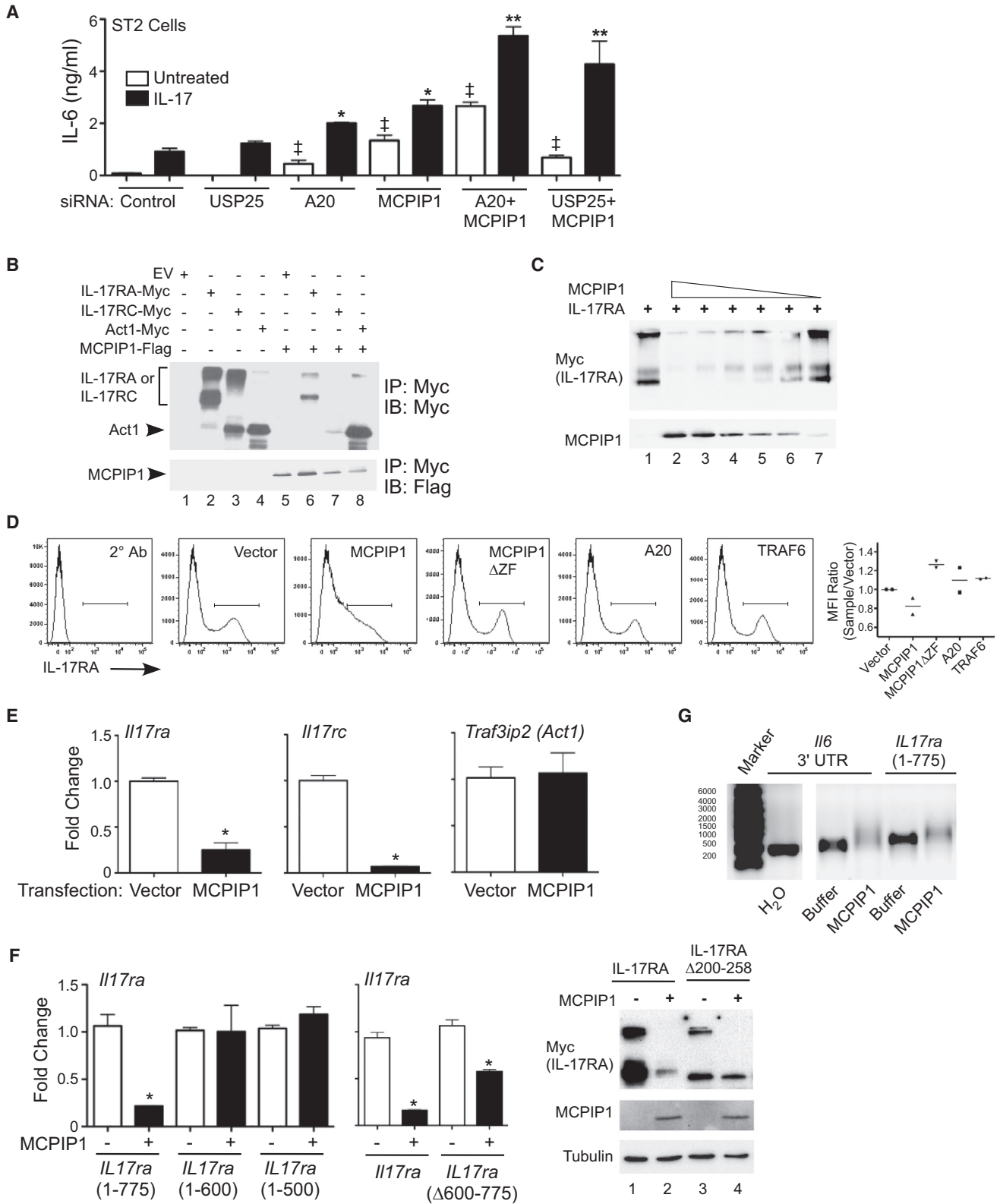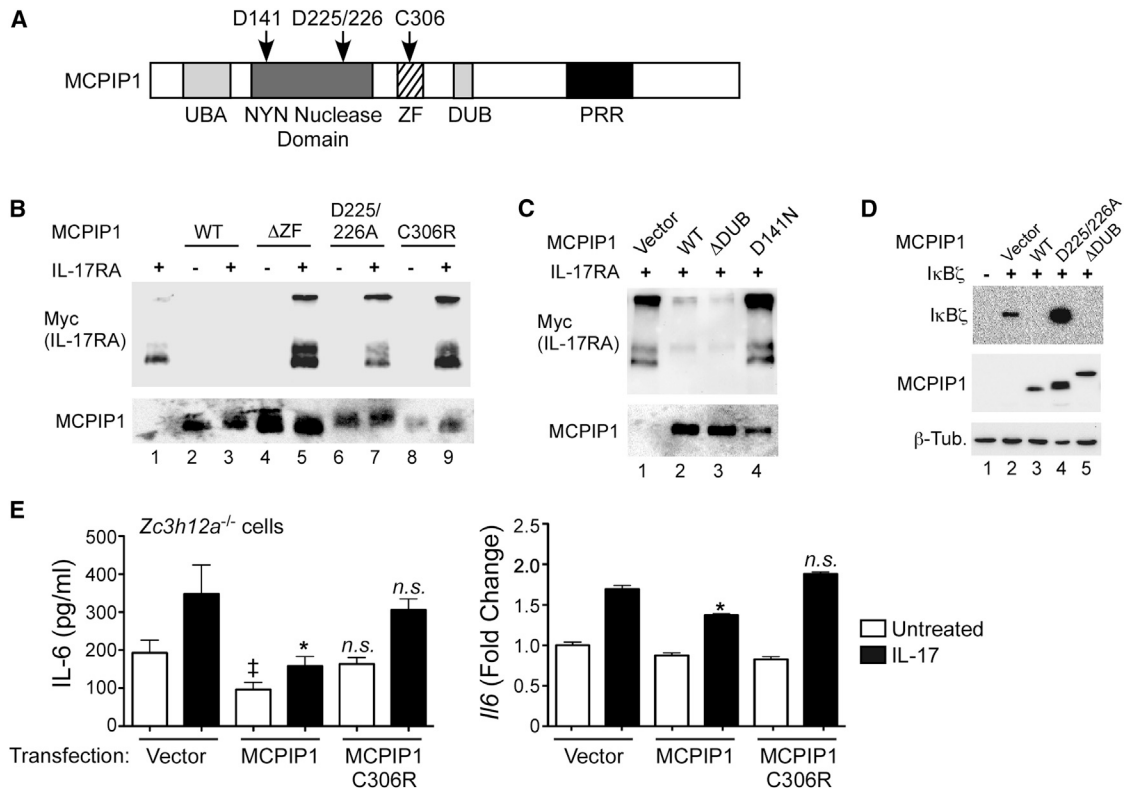
Figure 6. MCPIP1 Induces Degradation of IL-17R Subunit but Not Act1 mRNA Transcripts

(A) ST2 cells were transfected with indicated siRNAs, treated ± IL-17 for 3 hr, and analyzed for IL-6 by ELISA. *p < 0.05 versus IL-17-treated control siRNA. ‡p < 0.05 versus unstimulated control. Data presented as mean ± SEM.

Figure 7. Degradation of IL-17RA by MCPIP1 Requires RNase but Not DUB Activity

(A) Diagram of MCPIP1 subdomains. Residue designations are for the human homolog. Abbreviations are as follows: UBA, ubiquitin association domain; NYN, Nedd4-BP1, YacP nuclease; ZF, zinc finger; DUB, deubiquitinase domain; PRR, proline-rich region.

(B and C) HEK293T cells were transfected with plasmids encoding human MCPIP1 or mutants with Myc-tagged murine IL-17RA. After 24 hr, whole cell lysates were immunoblotted for Myc (top) and MCPIP1 (bottom).

(D) HEK293T cells were transfected with IκBζ with a 3′ UTR with MCPIP1 and the indicated mutants. Lysates were analyzed by immunoblotting for IκBζ (top), MCPIP1 (middle), or β-tubulin (bottom). Note that the ΔDUB mutant (Δ371–385) is GFP-tagged and migrates as a larger band.

(E) $Zc3h12a^{-/-}$ cells were transfected with vector, MCPIP1, or the MCPIP1.C306R mutant. Cells were treated with IL-17 for 3 hr. IL-6 was assessed by ELISA and $Il6$ assessed by qPCR. *$p < 0.05$ versus IL-17-treated vector control. ‡$p < 0.05$ versus unstimulated vector control. n.s., not significant.

Data presented as mean ± SEM throughout. Data expressed as fold-change relative to untreated vector control. Experiments were performed a minimum of twice.

because CD4-specific *Zc3h12a*-knockout mice survive significantly longer than those with a complete MCPIP1 deficiency, MCPIP1 evidently functions in both T cells and non-T cell types. In this regard, IL-17 signaling occurs dominantly in non-hematopoietic cells. In contrast to the TCR pathway, MCPIP1 is induced during IL-17R signaling to act as a break. Thus, MCPIP1 restricts

both IL-17 production in lymphocytes through inhibition of TCR signaling and IL-17 responsiveness in non-hematopoietic cells by virtue of its role as a feedback inhibitor.

TRAF3 and A20 bind to IL-17RA at a domain associated with negative feedback regulation (Garg et al., 2013; Zhu et al., 2010). In assessing whether MCPIP1 associates with IL-17RA

(B) HEK293T cells were transfected with murine Flag-tagged MCPIP1 together with Myc-tagged murine IL-17RA, IL-17RC, and Act1. After 24 hr, lysates were immunoprecipitated with anti-Myc Abs and immunoblotted for Myc and Flag. Arrows indicate IL-17RA, IL-17RC, or Act1.

(C) HEK293T cells were transfected with varying concentrations of Flag-MCPIP1 with Myc-IL-17RA. Lysates were immunoblotted for Myc or Flag.

(D) Left: HEK293T cells were transected with mIL-17RA and the indicated genes (vector, A20, TRAF6, MCPIP1, MCPIP1ΔZF). After 24 hr, cells were stained with APC-tagged α-IL-17RA Abs and APC-tagged 2° Ab. Right: MFI within IL-17RA⁺ gates, depicted as the ratio of the MFI of each sample relative to vector control. Data for two independent experiments is shown.

(E) HEK293T cells were transfected with MCPIP1 and the indicated constructs. After 24 hr, mRNA was assessed by qPCR. Data are expressed as fold-change relative to vector-transfected controls. *$p < 0.05$ versus corresponding samples without MCPIP1.

(F) HEK293T cells were transfected with MCPIP1 and the indicated IL-17RA mutants. After 24 hr, mRNA was assessed by qPCR. Data are expressed as fold-change relative to $Il17ra$(1-775)-transfected control. *$p < 0.05$ versus corresponding samples without MCPIP1. HEK293T cells were transfected with the indicated constructs. Left: After 24 hr, mRNA was assessed as in (E). Right: Whole cell lysates were analyzed by immunoblotting as in (B). Note that the numbers used for the mutant in the right panel represent amino acids.

(G) In vitro transcribed mRNAs encoding $Il6$ 3′ UTR or $Il17ra$ (nucleotides 1–775) were incubated with water, buffer, or recombinant MCPIP1 for 1 hr at 30°C. Transcripts were analyzed on a denaturing agarose gel. Panels are derived from the same gel image.

Data presented as mean ± SEM throughout.

and IL-17RC, we made the unexpected observation that MCPIP1 instead triggered their degradation, driven at the level of mRNA expression. MCPIP1 degrades *Il6* through its 3′ UTR (Matsushita et al., 2009), which we also demonstrate for *Nfkbiz* (encoding IκBζ). Similarly, MCPIP1 targets c-Rel and *Il2* via the 3′ UTR (Li et al., 2012; Uehata et al., 2013). In contrast, the minimal *Il17ra* sequence targeted by MCPIP1 is located in the 5′ region of its mRNA. Because MCPIP1 also targets TNFR and TLR4, MCPIP1 might limit inflammatory signaling via degradation of inflammatory receptors. Regulation of mRNA stability might provide a potential therapeutic avenue for diseases where IL-17 activity is relevant. For example, chlorpromazine hydrochloride, an anticholinergic agent commonly used as an antipsychotic drug, inhibits LPS-induced IL-6 by blocking Arid5a, an RNA binding protein that stabilizes the *Il6* transcript (Masuda et al., 2013).

Although the finding that MCPIP1 degrades *Il17ra* was intriguing, we have not yet identified a setting where IL-17RA expression is decreased in vivo. Generally, *Il17ra* mRNA turnover is low, due in part to the absence of destabilizing sequences in the 3′ UTR, and it is likely that subsequent translation is also at a low steady-state level. MCPIP1 was recently shown to act primarily on transcripts undergoing active translation (i.e., in polysomes) (Mino et al., 2015). Only a few studies have reported dynamic regulation of IL-17RA; we and others showed that IL-17RA on T cells is induced by IL-21 and IL-15 (Lindemann et al., 2008; Zeng et al., 2005). IL-17RA was also shown to be up-regulated in neuroglial cells in vitro after immunization with CFA (Liu et al., 2014a), which we confirmed in vivo in this study. Despite evidence for increased *Il17ra* transcript levels in spinal cord, surface IL-17RA levels did not seem to be affected, indicating that this mechanism probably does not account for the enhanced pathology in *Zc3h12a*$^{+/-}$ mice. However, *Zc3h12a*$^{-/-}$ mice have elevated levels of IL-17A due to enhanced Th17 cell differentiation, and IL-17RA internalizes upon IL-17 signaling (Jeltsch et al., 2014; Lindemann et al., 2008); one could speculate that elevated IL-17RA was not observed in *Zc3h12a*$^{+/-}$ mice because its surface expression is offset by accelerated ligand-mediated internalization. Moreover, we did not evaluate IL-17RC levels in this study, nor can we rule out the possibility that there might be a transient change in IL-17RA expression at a time point that was not analyzed here. Similarly, we do not know whether changes in IL-17RA expression occur in other disease settings such as candidiasis. Certainly this topic warrants further investigation.

Emerging studies have revealed multiple checkpoints of IL-17 signaling. A20 and USP25 downregulate TRAF6, limiting NF-κB and MAPK activation (Garg et al., 2013; Zhong et al., 2012). C/EBPβ exerts negative effects on IL-17-dependent gene expression (Shen et al., 2009), and TRAF3 and TRAF4 block Act1 binding to the IL-17R or TRAF6, respectively (Gaffen et al., 2014; Zepp et al., 2012; Zhu et al., 2010). It is unclear why so many non-redundant mechanisms are invoked to constrain IL-17, but the requirement for multiple, concerted checkpoints in inflammation has been observed in numerous innate immune settings (Carpenter et al., 2014). One reason might relate to the kinetics of each inhibitor, permitting control at different stages after signal initiation. Although A20 and MCPIP1 are both induced by IL-17, their expression kinetics are dissimilar

(Garg et al., 2013). IκBα and A20 both inhibit NF-κB, but mathematical modeling of the TNFα pathway revealed that IκBα restricts activation of the first phase of NF-κB activation, whereas A20 controls the second phase (Werner et al., 2008). The application of computational modeling to the IL-17 pathway might lend insight into how negative regulatory signals are functionally integrated.

In summary, MCPIP1 negatively regulates signaling downstream of IL-17 on multiple fronts. One mechanism is through destabilization of *Il6* mRNA. IL-6 contributes to IL-17 activity in many settings, including candidiasis and EAE (Basu et al., 2008; Samoilova et al., 1998). However, not all the effects of MCPIP1 occur through regulation of IL-6 (Uehata et al., 2013). MCPIP1 also limits expression of effectors such as IκBζ that function positively in driving IL-17-dependent gene expression. Additionally, we uncovered a previously unrecognized capacity of MCPIP1 to degrade certain mRNA transcripts in a 3′ UTR-independent manner, including receptors such as the IL-17R and TLR4, although the relative importance of receptor regulation compared to MCPIP1's other activities so far appears to be minimal. The cumulative impact of MCPIP1 on these signaling effectors, and likely others yet to be discovered, results in a marked and biologically important constraint of IL-17-mediated signal transduction.

EXPERIMENTAL PROCEDURES

Cell Culture
ST2, primary fibroblasts, and HEK293T cells were cultured in α-MEM (Sigma) with 10% FBS, L-glutamine, and antibiotics (Invitrogen). OKF6-TERT2 cells (Dickson et al., 2000) were provided by J. Rheinwald (Brigham & Women's Hospital) cultured in Serum-Free Fibroblast media, 25 μg/ml Bovine Pituitary Extract, and 2 μg/ml EGF (Life Technologies). HEK293T cells were transfected by CaPO₄. ST2 and 1° fibroblasts were transfected with Fugene 6 or Fugene HD (Promega). IL-17 was from Peprotech and used at 100–200 ng/ml.

siRNA, Plasmids, and Luciferase Assays
ON-TARGETplus SMARTpool siRNAs were from Dharmacon. ST2 cells were transfected with 50 nM siRNA using DharmaFECT Reagent 1 (Dharmacon). Plasmids encoding mIL-17RA, IL-17RC, MCPIP1, and mutants were described (Kapoor et al., 2015; Liang et al., 2010; Maitra et al., 2007; Shen et al., 2009). IκBζ with 3′ UTR was provided by U. Siebenlist (NIH). The ΔDUB mutant (GFP-tagged) lacks residues 371–385 and retains full RNase and anti-Dicer activities. Luciferase assays were performed as described (Shen et al., 2006).

RNA Isolation and qPCR
RNA was isolated with RNeasy Mini Kits (QIAGEN). cDNA was generated with Superscript III First Strand kits (Invitrogen). Genes were measured by real-time RT-PCR (qPCR) using SYBR Green FastMix ROX (Quanta Biosciences) on a 7300 Real Time instrument (Applied Biosystems). Expression was normalized to *Gapdh*. Primers were from Super Array Biosciences or QuantiTect Primer Assays (QIAGEN).

ELISA, Immunoprecipitations, Histology, Flow Cytometry
Immunoblotting and immunoprecipitations were performed as described (Ho et al., 2010; Maitra et al., 2007). Antibodies α-A20, α-myc, and α-IκBζ were from Cell Signaling; α-MCPIP1, α-TRAF6, and α-Act1 from Santa Cruz Biotechnology; and α-HA and α-FLAG from Sigma. Blots were developed with a FluorChem E imager (Protein Simple). Abs against IL-17RA were from Amgen (clone M751). ELISA kits were from eBioscience. Histology was performed by the University at Buffalo Histology Core and imaged on an EVOS FL microscope system (Life Technologies). For flow cytometry, CNS cells

were stained with Abs from eBioscience or BD and analyzed on a FACS Fortessa with FlowJo (Tree Star).

Protein Purification and In Vitro RNA Degradation

For expression, 7.5 × 10⁶ HEK293T cells were transfected with His-MCPIP1 (human). 48 hr later, cells were sonicated in 50 mM Tris-HCl (pH 8.3)/10% glycerol, 20 mM imidazole, 5 mM β-mercaptoethanol, and protease inhibitor cocktail (Calbiochem). Lysates were centrifuged at 15,000 rpm for 30 min and passed over an imidazole gradient using HiTrap chelating HP (GE Healthcare) charged with Ni sulfate. Fractions were dialyzed against 50 mM Tris-HCl (pH 8.3)/150 mM NaCl/3 mM DTT and concentrated with Amicon Ultra centrifugal units-30K (Millipore). MCPIP1 was confirmed in fractions by immunoblotting with α-His. RNAs encoding IL-17RA (1–775), Act-1, or IL-6 3′ UTR (2 μg) in the pCR2.1 vector were synthesized with TranscriptAid T7 High Yield Transcription Kits (Thermo Scientific). Transcripts were incubated with MCPIP1 (~2 μg) in 25 mM HEPES, 50 mM potassium acetate, 5 mM DTT, and RNasin (40 U) (Promega) for 1 hr at 30°C. RNA was analyzed on denaturing 1% agarose (Lin et al., 2013, 2014; Matsushita et al., 2009).

Mice

Mice were age and sex matched on the C57BL/6 background. WT mice were from The Jackson Laboratory. In experiments with *Zc3h12a*⁺/⁻ mice, littermates were used. *Il17ra*⁻/⁻ were from Amgen and bred in-house. Protocols were approved by the University of Pittsburgh IACUC and adhered to guidelines in the Guide for the Care and Use of Laboratory Animals of the NIH.

Intranasal IL-17 Delivery

Mice were treated intranasally with carrier-free IL-17 (R&D Systems) (300–500 ng). BALF was obtained with 0.5 ml PBS/0.5 mM EDTA followed by a 4 ml lavage. Supernatants from first lavage were used in ELISA and harvests combined for FACS. Cells were stained with α-CD11b (BD), α-Gr-1 (BioLegend), and α-F4/80 (eBiosciences). Left lobe of lung was used for qPCR. Flow cytometry was performed on a Becton Dickinson LSR II and analyzed by FlowJo (Tree Star).

Candidiasis

Oral candidiasis was performed by sublingual inoculation of *C. albicans* (CAF2-1) for 75 min (Conti et al., 2009). RNA was prepared from tongue by dissociation on a GentleMACS with M-tubes (Miltenyi Biotec). For systemic candidiasis, mice were injected i.v. with 1-2 × 10⁵ *C. albicans* yeast cells in 100 μl PBS. Kidney was homogenized in C-tubes in 2 ml PBS and plated on YPD-AMP. Anti-IL-6R Abs from Genentech were used at 20 mg/kg on days −2, −1, and +1 relative to infection.

Experimental Autoimmune Encephalomyelitis

Female mice were immunized subcutaneously in 4 sites on the back with 100 μg myelin oligodendrocyte glycoprotein (MOG) peptide (aa 35–55) emulsified with Complete Freund's Adjuvant with 1 mg *M. tuberculosis* strain H37Ra (DIFCO). Mice received 100 ng pertussis toxin (List Biological Laboratories) i.p. on day 0. Mice were assessed daily by blinded assessors and scored as follows: 1, flaccid tail; 2, impaired righting reflex and hindlimb weakness; 3, partial hindlimb paralysis; 4, complete hindlimb paralysis; 5, hindlimb paralysis with partial forelimb paralysis; 6, moribund.

Statistics

Data were analyzed by Kaplan-Meier, ANOVA, Mann-Whitney, or unpaired Student's t test by GraphPad Prism.

SUPPLEMENTAL INFORMATION

Supplemental Information includes six figures and can be found with this article online at http://dx.doi.org/10.1016/j.immuni.2015.07.021.

AUTHOR CONTRIBUTIONS

A.V.G., N.A., K.C., J.A.C., N.W., H.R.C., G.H.M., T.S., E.C.C., and P.S.B. did experiments. A.V.G., N.A., K.C., J.A.C., P.G., T.E.S., J.K.K., M.J.M., P.E.K., and S.L.G. designed experiments and aided with data analysis. A.V.G. and S.L.G. wrote the manuscript.

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