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The E3 ligase Itch and deubiquitinase Cyld co-operatively regulate Tak1 and inflammation

Neesar Ahmed¹, Minghui Zeng¹, Indrajit Sinha¹, Lisa Polin¹, Wei-Zen Wei¹, Chozhavendan Rathinam², Richard Flavell³, Ramin Massoumi⁴, and K Venuprasad¹

¹Karmanos Cancer Institute, Departments of Oncology, Immunology & Microbiology, Wayne State University School of Medicine, Detroit, MI 48201, USA

²Department of Genetics & Development, Columbia University Medical Center, New York, NY 10032, USA

³Department of Immunobiology, Yale University School of Medicine, New Haven, CT 06520, USA

⁴Department of Laboratory Medicine, Lund University, Malmö, Sweden

Abstract

Chronic inflammation has been strongly associated with tumor progression, but the underlying mechanisms remain elusive. Here we demonstrate that E3 ligase Itch and deubiquitinase Cyld form a complex *via* the interaction through ‘WW-PPXY’ motifs. The Itch-Cyld complex sequentially cleaved K63-linked ubiquitin chains and catalyzed K48-linked ubiquitination on the kinase Tak1 to terminate inflammatory tumor necrosis factor signaling. Reconstitution of wild-type Cyld but not mutant Cyld(Y485A), which cannot associate with Itch, blocked the sustained Tak1 activation and proinflammatory cytokine production by *Cyld*^{-/-} bone marrow-derived macrophages. Itch or Cyld deficiency resulted in chronic production of tumor-promoting cytokines by the tumor-associated macrophages and aggressive growth of lung carcinoma. Thus, we have uncovered an Itch-Cyld mediated regulatory mechanism in innate inflammatory cells.

INTRODUCTION

Accumulating evidence indicates that tumor progression is governed not only by genetic changes in cancer cells, but also by epigenetic and environmental factors. Chronic infection and subsequent inflammation are considered critical environmental and epigenetic factors contributing to tumor progression^{1, 2}. Primary examples are gastric cancer and *Helicobacter pylori* infections, hepatocellular carcinoma and viral hepatitis, colitis-associated cancer, and

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Correspondence should be addressed to K.V. (poojaryv@karmanos.org).

Contributions

N.A., M.Z., and I.S. did the experiments and analyzed data; L.P. assisted with *in vivo* tumor studies; W.W. assisted in manuscript preparation; C.R., R.F. provided critical reagents and mouse model; R.M. provided critical mouse model and assisted in manuscript preparation; K.V. designed the experiments, analyzed data and wrote the manuscript.

Competing financial interests

The authors declare no competing financial interests.

pancreatic cancer associated with chronic pancreatitis¹. Similarly, chronic obstructive pulmonary disease (COPD), cigarette smoking, exposure to asbestos and silica are all associated with lung inflammation and lung carcinoma^{3–5}. Inflammatory cells found in the tumor microenvironment, particularly tumor-associated macrophages (TAMs), constitute an important interface between tumor cells and the immune system⁶. Production of pro-inflammatory cytokines such as tumor necrosis factor (TNF), interleukin 6 (IL-6) and IL-1 β , by TAMs not only trigger pro-survival signals in tumor cells, but also support their growth and metastasis^{6, 7}. However, the molecular mechanisms that regulate inflammation and tumor progression remain unclear.

Post-translational modifications mediated by ubiquitin conjugation have emerged as a key regulatory mechanism in the immune cells^{8, 9}. The ubiquitin signal is interpreted based on the number of attached ubiquitin chains and the topology of their linkage. A polyubiquitin chain is formed when one of the seven lysines within ubiquitin is linked to the C-terminal glycine of another ubiquitin. Although ubiquitin contains seven lysine residues, linkage generally occurs *via* either K48 or K63. K48-linked polyubiquitination predominantly targets proteins for proteasomal degradation, whereas K63-linked polyubiquitination results in non-proteasomal modifications, such as subcellular localization or protein-protein interactions⁸. We and others have demonstrated HECT type E3 ligase Itch as a critical regulator of inflammation^{10, 11}. On a mouse C57BL/6 background, Itch deficiency results in the development of a late onset lymphoproliferation disorder and chronic pulmonary inflammation^{12, 13}. Itch possesses four WW domains that recognize the Pro-rich PPXY (PY) consensus sequence in their substrate targets¹⁴. Although it is clear that Itch regulates inflammation, no systematic study has linked Itch to tumorigenesis.

Similar to phosphorylation, protein ubiquitination is reversible, and removal of ubiquitin molecules is mediated by de-ubiquitinating (DUB) enzymes, such as Cyld¹⁵. Cyld is encoded by a NF- κ B-inducible gene¹⁶, inhibits NF- κ B and the kinase JNK by removing K63-linked polyubiquitin chains from signaling pathway molecules such as Bcl-3, TRAF2, TRAF6 and Nemo (also known as IKK- γ)^{17–21}. Cyld through its deubiquitinating function has been shown to regulate various types of human cancers, including lung, hepatocellular, colon and cervical cancers¹⁵. Cyld was also shown to negatively regulate tumor-promoting cytokine secretion by inflammatory cells²². Despite these important observations, the molecular mechanisms regulating Cyld function and its role in inflammation remain largely unknown. Here, we demonstrate that Itch interacts with Cyld and cooperatively regulates Tak1 and inflammation.

RESULTS

Augmented tumor growth in *Itch*^{-/-} and *Cyld*^{-/-} mice

We have demonstrated earlier that Itch deficiency results in elevated lung inflammation^{10, 11}. Similarly, *Cyld*^{-/-} mice exhibit inflammatory infiltrates in numerous tissues including lung, liver, spleen and the salivary glands²². Cyld deficiency resulted in increased susceptibility to spontaneous and chemically induced colitis and colon tumorigenesis^{22, 23}. Cyld was also shown to regulate lung inflammation^{24, 25} and Cyld expression is frequently downregulated in non-small cell lung carcinoma (NSCLC) cell

lines²⁶. Over-expression of the full-length but not the catalytically inactive mutant of Cyld in NSCLC cell lines significantly reduces cell growth, suggesting that Cyld acts as tumor suppressor gene for the progression of NSCLC and that its deubiquitin function is important for this mechanism²⁶. To determine the contribution of Itch and Cyld in inflammation and metastasis of lung carcinoma, we inoculated 1×10^6 Lewis Lung Carcinoma (LLC) cells into *Cyld*^{-/-}, *Itch*^{-/-} and control C57BL/6 mice intravenously *via* tail vein. Both *Itch*^{-/-} and *Cyld*^{-/-} mice exhibited higher mortality rate compared to wild-type mice (Fig. 1a). Histological analysis revealed aggressive tumor growth in the lungs of *Itch*^{-/-} and *Cyld*^{-/-} mice (Fig. 1b). Similarly, remarkable differences were seen in tumor multiplicity (Fig. 1c) and tumor size (Fig. 1d). To investigate the effect of Itch and Cyld deficiency on metastasis, we inoculated LLC cells subcutaneously (s.c.) and spontaneous lung metastasis was analyzed. A marked increase in the metastasis of LLC tumors was observed in *Itch*^{-/-} and *Cyld*^{-/-} mice (Supplementary Fig. 1). These data suggest that Itch and Cyld deficiency in the hosts result in elevated growth and metastasis of lung carcinoma.

Since proinflammatory cytokines secreted by TAMs have been associated with enhanced tumor growth and metastasis⁶, we analyzed cytokine expression by tumor infiltrated CD11b⁺ cells. Enhanced expression of TNF, IL-6 and IL-1 β was observed in both *Itch*^{-/-} and *Cyld*^{-/-} cells (Fig. 1e). Similarly, we have observed elevated expression of these cytokines from the whole tumor nodules isolated from the lungs of *Itch*^{-/-} and *Cyld*^{-/-} mice (data not shown). No significant difference in cytokine expression was observed in the lung tissues of naïve *Itch*^{-/-} and *Cyld*^{-/-} mice that were not inoculated with LLC cells (Fig. 1f), suggesting that Itch and Cyld negatively regulate TAM activity.

Itch interacts with Cyld *via* the WW-PPXY motifs

We previously reported that Itch regulated TNF-induced NF- κ B signaling by modulating cFLIP_L turnover²⁷. Cyld deubiquitin activity was shown earlier to interfere with the ubiquitination of TRAF molecules and Tak1, which are involved directly in the TNF-associated NF- κ B signaling pathway¹⁵. Since Itch and Cyld can regulate similar pathways, we tested whether these enzymes can cooperate to terminate NF- κ B signaling and inflammatory response during tumor growth and metastasis. To determine whether Itch and Cyld can directly interact with each other, we performed sequence analysis and found a highly conserved Cyld 'PPXY' motif (Fig. 2a) by which it could interact with Itch 'WW' domains. We tested this interaction by transiently transfecting 293T cells with Flag-Itch and Myc-Cyld. Cell lysates were immunoprecipitated using control mouse IgG or antibodies against Flag or Myc. Antibody against Myc immunoprecipitated Flag-Itch and *vice versa*, suggesting that Itch physically interacts with Cyld (Fig. 2b). Further, we generated a fusion protein of glutathione S-transferase (GST) and Itch (GST-Itch) and performed pull-down assay using lysates of 293T cells transfected with Myc-Cyld, GST-Itch precipitated Myc-Cyld, but not GST alone, confirming the association (Supplementary Fig. 2). We further tested whether the interaction is direct by using GST-Itch and His-Cyld (expressed in *Escherichia coli*). GST-Itch precipitated His-Cyld in the pull-down assay confirming a direct interaction between Itch and Cyld (Fig. 2c).

To determine if this interaction occurs endogenously in primary cells, we stimulated bone marrow derived macrophages (BMDMs) with TNF for 30 min followed by immunoprecipitation experiments using antibodies against Itch and Cyld. Itch co-immunoprecipitated with Cyld (Fig. 2d). Itch and Cyld interaction in BMDMs seems to be stimulation dependent as we did not detect the association in unstimulated cells (data not shown). To test whether Itch-Cyld interaction occurs *via* the 'PPXY' motif, we generated Y to A mutant Cyld (Cyld(Y485A)) by site-directed mutagenesis. The Cyld(Y485A) mutation disrupted Itch-Cyld interaction (Fig. 2e). Taken together these data collectively indicate that Itch forms a complex with Cyld *via* its interaction through 'PPXY' motif.

Itch targets Tak1 for K48-linked ubiquitination

Next, we sought to understand the role of Itch-Cyld complex in the regulation of inflammatory response. Inflammatory stimuli driven signaling converge at the I κ B kinase (IKK) complex, a trimeric holoenzyme comprised of catalytic subunits IKK α , IKK β , and the regulatory subunit Nemo²⁸. Tak1 is a key intermediate that transmits signals from receptor complexes to IKK. Tak1 associates with TNF-receptor associated factors TRAF2 or TRAF6 following stimulation with TNF or Toll-like receptor ligands and IL-1, respectively, to initiate a signaling cascade. Both TRAF2 and TRAF6 are RING domain ubiquitin ligases that catalyze K63-linked polyubiquitin chains, which results in Tak1 autophosphorylation²⁹. Activated Tak1 phosphorylates IKK β within the activation loop, leading to the activation of IKK³⁰. IKK in turn phosphorylates I κ B α , resulting in NF- κ B nuclear translocation and transcription of inflammatory target genes²⁸. Tak1 activation is transient and is rapidly inactivated, which is important for preventing prolonged inflammatory responses³¹⁻³³. Cyld was previously shown to cleave K63-linked polyubiquitin chain on Tak1²³. Because Itch deficiency resulted in persistent IKK and Jnk kinase activation³⁴, similar to Cyld deficiency¹⁷⁻²¹, we reasoned that Itch may participate in terminating Tak1 activation by K48-linked polyubiquitination. To test this notion, we co-expressed Flag-Tak1 with Myc-Itch and either Hemagglutinin (HA) tagged Ub(WT), Ub(K48) [in which all the lysine residues, except K48, are mutated], or Ub(K63) [in which all the lysine residues, except K63, are mutated]. Slow-migrating high molecular weight polyubiquitinated forms of Tak1 was observed when Itch and Tak1 were co-expressed with Ub(WT) (Fig. 3a, lane1). Interestingly, Itch catalyzed Tak1 polyubiquitination when co-expressed with Ub(K48) (lane3), but not with Ub(K63) (lane5) suggesting that Itch targets Tak1 for K48-linked but not K63-linked ubiquitination. To confirm the specificity of Itch as the E3 ligase, we generated an Itch(C830A) mutant in which the conserved cysteine is mutated to alanine³⁵. Expression of the Itch(C830A) mutant with either Ub(WT) or Ub(K48) did not result in Tak1 ubiquitination (Fig. 3a, lanes 2 and 4). To further confirm that Itch targets Tak1 for K48-linked polyubiquitination, we utilized Ub(K48R) in which only K48 was mutated to arginine. Co-expression of Ub(K48R) and Itch failed to polyubiquitinate Tak1 (Fig. 3b). Next, we tested whether another functionally related E3 ligase, Cbl-b⁸ ubiquitinates Tak1. Co-expression of Cbl-b with Tak1 did not result in Tak1 ubiquitination (Supplementary Fig. 3), suggesting that Itch is the specific ligase for Tak1.

Itch-Cyld complex edits Tak1 ubiquitination

Because Cyld cleaves K63-linked ubiquitination of Tak1²³, we hypothesized that the 'Itch-Cyld' complex sequentially cleaves K63-linked ubiquitin chain and catalyzes K48-linked ubiquitination to deactivate Tak1 and terminate NF- κ B signaling. Co-expression of TRAF2 with Tak1 and Ub(K63) resulted in Tak1 polyubiquitination (Fig. 3c, lane1). As expected the RING deletion mutant of TRAF2 that lacks the ligase activity³⁶ failed to ubiquitinate Tak1 (Fig. 3c, lane2). Inclusion of Cyld(WT) (Fig. 3c, lane3) but not Cyld(C601A) mutant (Fig. 3c, lane4) that lacks the DUB activity³⁷ resulted in diminished Tak1 ubiquitination suggesting that Cyld deubiquitinated Tak1. Similarly, Cyld(Y485A) mutant was defective in deubiquitinating Tak1 (Fig. 3c, lane5). Itch failed to ubiquitinate Tak1 in the presence of Cyld, TRAF2, and Ub(K63) (Fig. 3c, lane6), but in a similar combination co-expression of Ub(K48) resulted in Tak1 ubiquitination (Fig. 3c, lane7). This result suggests that following cleavage of K63-linked polyubiquitin chain, Itch catalyzes K48-linked polyubiquitination. The possibility of cross reactivity of antibodies against Xpress (HX) and Flag in these experiments are ruled out as we obtained single specific bands (Supplementary Fig. 4).

To further confirm these results we utilized Ub(K48R) (in which all lysines, except K48, are intact) and Ub(K63R) (in which all lysines, except K63, are intact). Expression of TRAF2 with Ub(K48R) (Fig. 3d, lane 2) but not Ub(K63R) (Fig. 3d, lane 1) resulted in Tak1 polyubiquitination. As expected RING deletion mutant of TRAF2³⁶ was defective in catalyzing ubiquitin chain on Tak1 (Fig. 3, lane 3). Cyld(WT) (Fig. 3d, lane 4) but not Cyld(C601A) mutant³⁷ deubiquitinated Tak1 (Fig. 3d, lane 5). Similarly, Cyld(Y485A) mutant was defective in deubiquitination (Fig. 3d, lane 6). Itch was unable to ubiquitinate Tak1 (Fig. 3d, lane 7) in the presence of Ub(K63R) but in a similar combination co-expression of Ub(K48R) resulted in Tak1 polyubiquitination (Fig. 3d, lane8). Similar results were obtained when TRAF6 was used instead of TRAF2 (Supplementary Fig. 5). To further demonstrate that Cyld-mediated cleavage of K63-linked ubiquitin chain formed by TRAF2 is a prerequisite for Itch-mediated K48-linked ubiquitination, we used Flag-Ub(K63) and HA-Ub(K48). Itch failed to form K48-linked ubiquitin chain on Tak1 without Cyld-mediated removal of K63-linked ubiquitin chain (Fig. 3e). These results suggest that Itch and Cyld cooperatively 'switch' K63-linked to K48-linked ubiquitination.

We also tested whether additional deubiquitinases, A20 and Cezanne^{38, 39}, which deubiquitinate the signaling molecule RIP1, likewise regulate Tak1 ubiquitination. We co-expressed Tak1, Ub(K63), and TRAF2 along with either A20 or Cezanne. Both A20 and Cezanne failed to deubiquitinate Tak1 suggesting the substrate specificity of Cyld (Supplementary Fig. 6). Next, we analyzed whether Itch-mediated K48-linked polyubiquitination results in Tak1 degradation. Co-expression of HA-Tak1 with increasing Flag-Itch but not Itch(C830A) mutant resulted in reduced Tak1 abundance, suggesting that Itch regulates Tak1 turnover (Fig. 4a). Further, MG132 a proteasome inhibitor blocked Itch-mediated degradation of Tak1 in a similar experiment (Fig. 4b). This suggests that Itch-mediated K48-linked ubiquitination promotes proteasomal degradation of Tak1.

Tak1 activation in *Itch*^{-/-} and *Cyld*^{-/-} BMDMs

Subsequently, we investigated the effect of Itch and Cyld deficiency on Tak1 activation in BMDMs following treatment with TNF. The cell lysate was immunoprecipitated using antibody against TRAF2 and immunoblotted with antibody against phospho-Tak1 to test TRAF2-associated Tak1 activation. Tak1 phosphorylation peaked within five minutes following stimulation in wild-type macrophages and the phosphorylation was reduced to minimal within thirty minutes, however, we have observed sustained Tak1 phosphorylation in *Itch*^{-/-} and *Cyld*^{-/-} BMDMs (Fig. 5a). We reprobated the membranes with Tak1-specific antibody to analyze total TRAF2-associated Tak1. Similar to Tak1 phosphorylation, association of Tak1 with TRAF2 was transient in wild-type macrophages; however, Itch and Cyld deficiency prolonged the association. To analyze the effect of Itch and Cyld deficiency on total cellular Tak1 abundance, we immunoblotted the cell lysate with antibody against Tak1. We observed only a marginal decrease in the total Tak1 protein in wild-type BMDMs stimulated with TNF. This finding suggests that Itch-Cyld complex degrades the Tak1 that is associated with TRAF complex but not the entire pool of Tak1 within the cell during an inflammatory response.

Because Tak1 activation is directly linked to pro-inflammatory cytokine secretion, we analyzed the expression of IL-6, TNF and IL-1 β in BMDMs after stimulation with TNF. Expression of proinflammatory cytokine mRNA was sustained until 12 hours in *Itch*^{-/-} and *Cyld*^{-/-} BMDMs (Fig. 5b). We also analyzed the cytokine concentrations in the culture supernatant by ELISA. Itch and Cyld deficiency resulted in elevated secretion of proinflammatory cytokines (Fig. 5c). This result provides evidence that cleavage of K63-linked polyubiquitination is not sufficient to deactivate Tak1 and downstream effector function, rather sequential Itch-mediated K48 linked ubiquitination is essential. Similarly, sustained Tak1 phosphorylation and elevated cytokine mRNA expression were observed in *Itch*^{-/-} and *Cyld*^{-/-} BMDMs stimulated with lipopolysaccharide (LPS) (Supplementary Fig. 7).

To test whether inhibiting Tak1 activation attenuates cytokine production, we used (5Z)-7-Oxozeanol, a selective inhibitor of Tak1⁴⁰. BMDMs were pre-incubated for 30 minutes prior to TNF stimulation with indicated concentrations of (5Z)-7-Oxozeanol, which decreased cytokine mRNA abundance in a dose-dependent manner (Fig. 6a). Similar results were obtained when BMDMs were stimulated with LPS in the presence of (5Z)-7-Oxozeanol (Supplementary Fig. 8). Also, pre-incubation of BMDMs with (5Z)-7-Oxozeanol inhibited sustained Tak1 phosphorylation observed in *Itch*^{-/-} and *Cyld*^{-/-} BMDMs (Fig. 6b). Next, we tested whether ectopic expression of a catalytically inactive dominant-negative dnTak1 (Tak1(K63W))⁴¹ inhibits elevated cytokine production by *Itch*^{-/-} and *Cyld*^{-/-} BMDMs. Expression of dnTak1 in primary BMDMs inhibited cytokine production by *Itch*^{-/-} and *Cyld*^{-/-} BMDMs (Fig. 6c-d). These data collectively suggest that sustained Tak1 activation in *Itch*^{-/-} and *Cyld*^{-/-} BMDMs results in chronic production of proinflammatory cytokines.

Itch and Cyld enzyme activity regulates Tak1

To determine the functional relevance of Tak1 regulation by Itch and Cyld, we reconstituted *Itch*^{-/-} MEFs with Itch(WT) and the ligase deficient Itch(C830A) mutant. As expected,

reconstitution of *Itch*^{-/-} MEFs with *Itch*(WT) but not *Itch*(C830A) mutant normalized Tak1 activation (Fig. 7a). Similarly, IL-6 production by *Itch*^{-/-} MEFs reconstituted with *Itch*(WT) but not *Itch*(C830A) mutant was comparable to wild-type MEFs (Fig. 7b). This result is consistent with the finding that *Itch*(C830A) mutant failed to ubiquitinate Tak1 (Fig. 3a). We also reconstituted *Cyld*^{-/-} MEFs with *Cyld*(WT), *Cyld*(C601A) that lacks the DUB activity³⁷ and *Cyld*(Y485A) mutant that fails to interact with *Itch*. Reconstitution of *Cyld*^{-/-} MEFs with *Cyld*(WT) restored transient Tak1 activation (Fig. 7c–d) and normalized IL-6 production. However, *Cyld*(C601A) and *Cyld*(Y485A) mutants were impaired in the termination of Tak1 activation. Collectively these data indicate that DUB function of *Cyld* and its interaction with *Itch* is essential for the termination of Tak1 and inflammatory cytokine production.

To further confirm the role of *Itch* and *Cyld* interaction for terminating Tak1 activation in macrophages, we reconstituted *Cyld*^{-/-} BMDMs with *Cyld*(WT) or *Cyld*(Y485A) mutant that fails to interact with *Itch*. Expression of *Cyld*(WT) but not *Cyld*(Y485A) mutant restored transient Tak1 activation (Fig. 8a). Similarly, wild-type *Cyld* almost completely reverted elevated cytokine production by *Cyld*^{-/-} BMDMs; however, expression of *Cyld*(Y485A) mutant had only minimum effect (Fig. 8b–c). These data clearly suggest that *Itch* and *Cyld* cooperatively regulate cytokine production by the inflammatory cells.

DISCUSSION

In this study, we demonstrate that *Itch* forms a ubiquitin-editing complex with *Cyld* *via* the interaction through WW-PPXY motifs. *Itch*-*Cyld* complex promotes the transition of K63-linked to K48-linked ubiquitination by sequentially cleaving K63-linked ubiquitin chain and by catalyzing K48-linked ubiquitination on Tak1. During the early phase of TNF- or TLR-induced inflammatory response RING type E3 ligases TRAF2 and TRAF6 promote K63-linked ubiquitination within the kinase domain²⁹, promoting phosphorylation of Tak1 that leads to IKK-NF- κ B activation and transcription of proinflammatory cytokine gene expression⁴². We propose that the combined activity of *Itch* and *Cyld* shifts Tak1 ubiquitination from K63-linked toward K48-linked chains that triggers the proteasomal degradation of Tak1, thus terminating the inflammatory response. *Itch*-*Cyld* association was detected only following stimulation of BMDMs with TNF suggesting the requirement of extracellular signals. At present the molecular mechanisms that regulate *Itch*-*Cyld* complex formation remain unclear. One possibility is that *Cyld* expression is upregulated by TNF and IL-1 β , *via* IKK-NF- κ B pathway^{16, 24, 25} triggers its association with *Itch*. Alternatively, *Itch*-*Cyld* association could be regulated by NF- κ B-dependent phosphorylation mediated mechanisms. Such a phenomenon has recently been demonstrated for the recruitment of *Itch* to A20 complex by TAXIBP1^{34, 43}.

The molecular basis for the chronic multi-organ inflammatory disorder observed in *Itch*^{-/-} mice has been attributed to biased T_H2 differentiation due to the defect in the degradation of JunB¹³. Our previous studies have shown that *Itch* also regulates T cell tolerance, as *Itch*^{-/-} T cells are resistant to anergy induction⁴⁴ and TGF- β mediated suppression¹¹. Our present results indicate a critical role for *Itch* in the regulation innate immune cells *via* targeting Tak1 for proteasomal degradation. These findings along with recent reports that *Itch* is a

component of A20, TAXIBP1 and RNF11 complex^{34, 43} raise the possibility that Itch has more prominent role as a negative regulator of inflammatory signaling in the innate immune cells.

Itch seems to be a specific ligase that targets Tak1 for K48-linked ubiquitination as Cbl-b a functionally related E3 ligase⁸ which also regulates NF- κ B pathway⁴⁵ but failed to ubiquitinate Tak1. Similarly, several other DUBs including A20, Cezanne, USP4, and USP21 are shown to negatively regulate NF- κ B pathway³⁹. In our assay system we did not detect DUB activity of A20 or Cezanne towards Tak1, suggesting the specificity of Cyld. However, we do not completely rule out the involvement of additional DUBs or E3 ligases in the regulation of Tak1 and downstream signaling.

An additional observation from our studies is that *Itch*^{-/-} and *Cyld*^{-/-} TAMs produced elevated tumor promoting inflammatory cytokines. Consistently, *Itch*^{-/-} and *Cyld*^{-/-} mice exhibited aggressive growth and metastasis of transplanted LLC tumors. However, it is not clear whether the enhanced tumor growth observed is exclusively due to the chronic production of tumor promoting cytokines by TAMs. Aberrant T cell response in *Itch*^{-/-} and *Cyld*^{-/-} mice^{8, 10, 11, 13} may also contribute to tumor growth. Further detailed analysis is essential for a clear and better understanding of these mechanisms that could lead to innovative therapeutic targeting.

Consistent with our findings, cytokines produced by activated innate immune cells, particularly TAMs, have been associated with inflammation and cancer⁶. Clinical studies have shown that increased numbers of TAMs are correlated with angiogenesis, metastasis and poor prognosis⁶. Tumor-promoting role of TAMs is linked to NF- κ B-mediated production of pro-inflammatory cytokines, including TNF and IL-6⁷ supporting the notion that excessive, chronically produced pro-inflammatory cytokines contribute to tumor progression. Our findings have identified an unanticipated co-ordination between Itch and Cyld that restricts prolonged inflammatory signaling and secretion of tumor promoting inflammatory cytokines by the macrophages.

Our studies also show that Tak1 selective inhibitor, (5Z)-7-Oxozeaenol inhibits tumor promoting cytokine production by BMDMs. Since NF- κ B has emerged as a key internal factor that promotes inflammation and cancer^{1, 2}, several approaches have been designed to block NF- κ B, including inhibitors of IKK and proteasome inhibitors to inhibit I κ B degradation⁴⁶. Although proteasome inhibitors like bortezomib block NF- κ B in a variety of cells and are being used in clinical trials against multiple myeloma, the results of the phase I/II clinical trials indicate the drug has limited activity against advanced NSCLC^{47, 48}. In addition, complications have been reported after global inhibition of NF- κ B using genetic and pharmacological approaches⁴⁹. This highlights the requirement for more specific inhibitors to dampen inflammation and tumor progression. Our results provide a mechanistic insight into regulation of Tak1 for termination of inflammatory cytokine production which can be exploited therapeutically to target inflammatory cells in the tumor microenvironment. By targeting inflammatory cells, which are genetically normal and stable compared to genetically unstable carcinoma cells, development of drug resistance could be minimized. In

addition, a combination of conventional cytotoxic cancer therapy along with inhibitors that block inflammatory responses could potentially enhance current therapeutic strategies.

METHODS

Cell culture and transfection

293T, 293FT (Invitrogen R700-07) and Lewis Lung Carcinoma cells (LLC, ATCC CRL-1642) were cultured in DMEM supplemented with 10% FBS. All of the transient cell transfections were performed using Lipofectamine 2000 (Invitrogen) following manufacturer's instructions. MEFs were prepared from 12.5–14.5 day embryos. The genotyping of the *Itch*^{-/-} and *Cyld*^{-/-} MEFs was performed by PCR.

Animals

Cyld^{-/-} and *Itch*^{-/-} mice were described before^{12, 18}. Control C57BL/6 mice were purchased from Charles River Laboratory. All the mice were housed in microisolator cages in the barrier facility of Karmanos Cancer Institute. Animal experiments were performed in accordance with the guidelines of the Institutional Animal Care and Use Committee of Wayne State University/Karmanos Cancer Institute.

Plasmids and Constructs

Full length TRAF6 was amplified from human cDNA clone (MHS1010-7296061, Open Biosystems) and cloned into the vector pEF4/HisC (Invitrogen) between BamHI-EcoRI to create HX-TRAF6. Full length human Tak1 and mouse Tak1 were amplified from human and mouse cDNA clones (MHS1010-58135 and MMM1013-64018, Open Biosystems) and cloned into the vector pCMV-Tag2B between BamHI-EcoRI to create both human and mouse Flag-Tak1. HA-Ub(WT), HA-Ub(K63), HA-Ub(K48), GST-Itch, and Itch-C830A were described before³⁵. His-Cyld was created from myc-Cyld by subcloning into pET30a between BamHI-NotI. HX-Cyld(C601A) was created from HA-Cyld(C601A) (15507, addgene) by subcloning into pEF4/HisC between BamHI-NotI. HX-Cyld(Y485A) was created from Myc-Cyld by site-directed mutagenesis and subcloning into pEF4/HisC between BamHI-NotI. HX-TRAF2 was created from HA-Flag-TRAF2 (20229, Addgene) by subcloning into pEF4/HisC between BamHI-NotI. Myc tagged TRAF2 mutant lacking the Ring domain, amino acid residues 87–501 (Myc-TRAF2^Δ), was created by subcloning into pcDNA3.1/myc-His (-) A (Invitrogen) between BamHI-HindIII. Myc-A20 was created from pEGFP-C1-A20 (22141, Addgene) by subcloning into pcDNA3.1/myc-His (-) A between XhoI-EcoRI. HX-Cezanne was created from Flag-HA-OTUD7B (22550, Addgene) by subcloning into pEF4/HisC between EcoRI-NotI. All clones were sequence verified.

Antibodies and Reagents

Following antibodies were used in this study: antibody against c-Myc (sc-40, Santa Cruz), antibody against Flag (F1804, F7425, Sigma), antibody against Itch (611198, BD), antibody against Cyld (4495, Cell Signaling), antibody against Tak1 (sc-7162, Santa Cruz), antibody against phospho-Tak1 (4531, Cell Signaling), antibody against TRAF2 (sc-876, Santa Cruz), antibody against TRAF6 (sc-7221, Santa Cruz), antibody against actin (A5441, Sigma), antibody against Ubiquitin (sc-8017, Santa Cruz), antibody against Xpress(HX) (R910-25,

Invitrogen), antibody against HA (sc-805, Santa Cruz), antibody against V5 (R960-25, Invitrogen), normal Rabbit IgG (sc-2027) and normal mouse IgG (sc-2025). Tak1 inhibitor (5Z)-7-Oxozeaenol was purchased from Tocris Bioscience (Missouri, USA) and MG132 was from Calbiochem.

BMDMs

BMDMs were prepared from femurs and tibias of age matched WT, *Cyld*^{-/-} and *Itch*^{-/-} mice and cultured in DMEM supplemented with 15% FBS, 2mM L-glutamine, and 10 ng/ml GM-CSF (AF-315-03, Peprotech). BMDMs were stained with antibody against CD11b and subjected to flow cytometry to confirm the purity of macrophages (>90%). BMDMs were cultured for seven days (6 days with GM-CSF and 1 day without GM-CSF) prior to stimulation with 50 ng/ml TNF (RMTNFAI, Thermo Scientific) or 10 ng/ml LPS (*E. coli* J5, 437620, Calbiochem). In experiments that were performed along with pretreatment with Tak1 inhibitor (5Z)-7-Oxozeaenol, cells were pretreated for 30 min with varying concentrations of inhibitor as indicated before stimulation.

Lentiviral transduction

Expression clones were created using the Gateway cloning technology (Invitrogen) following the manufacturer's instructions. Dominant negative expression clone of mouse Tak1 was constructed by first PCR subcloning Tak1K63W (BamHI-EcoRI) into pENTR-3C entry vector (Invitrogen) and then LR recombined to pLenti6.2/N-Lumio/V5-DEST (Invitrogen). Expression clones for *Cyld* (BamHI-BamHI), *Cyld*(Y485A) (BamHI-BamHI), *Cyld*(C601A) (BamHI-NotI), *Itch* (BamHI-NotI) and *Itch*(Y485A) (BamHI-NotI) were similarly prepared by first PCR subcloning into pENTR-3C entry vector (Invitrogen) and then LR recombined to pLenti6.2/N-Lumio/V5-DEST (Invitrogen).

Lentiviral transduction of MEFs and BMDMs was performed using the ViraPower lentiviral expression system (Invitrogen). Briefly, 293FT cells were used to package and produce the viruses. BMDMs and MEFs were incubated with the virus-containing supernatants at 37°C overnight in a 5% CO₂ incubator. Successfully transduced cells were selected by further incubation for 48 h in medium containing 2 µg/ml Blasticidin before TNF treatment.

Lung metastasis assays

Sub-confluent LLC cells were trypsinized, washed three times with PBS, and filtered through a cell strainer to obtain single-cell suspension. Cells of >90% viability (Trypan Blue staining) were used. Seven weeks old mice were injected with 1×10⁶ cells *via* tail vein. Mice were sacrificed at indicated days and lungs were removed for histological examination while some mice were kept until death and survival data were obtained. Lung tumor multiplicity count, the number of incidences of tumors in lungs per mouse, was scored under the microscope after the lungs were paraformaldehyde fixed, paraffin embedded, sectioned and stained by hematoxylin and eosin staining. For spontaneous lung metastasis, 2×10⁶ cells were subcutaneously injected into the left flank of mice and tumor growth was monitored till the primary tumors reached a size of 2000 mm³ and after which mice were sacrificed to determine tumor multiplicity count.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

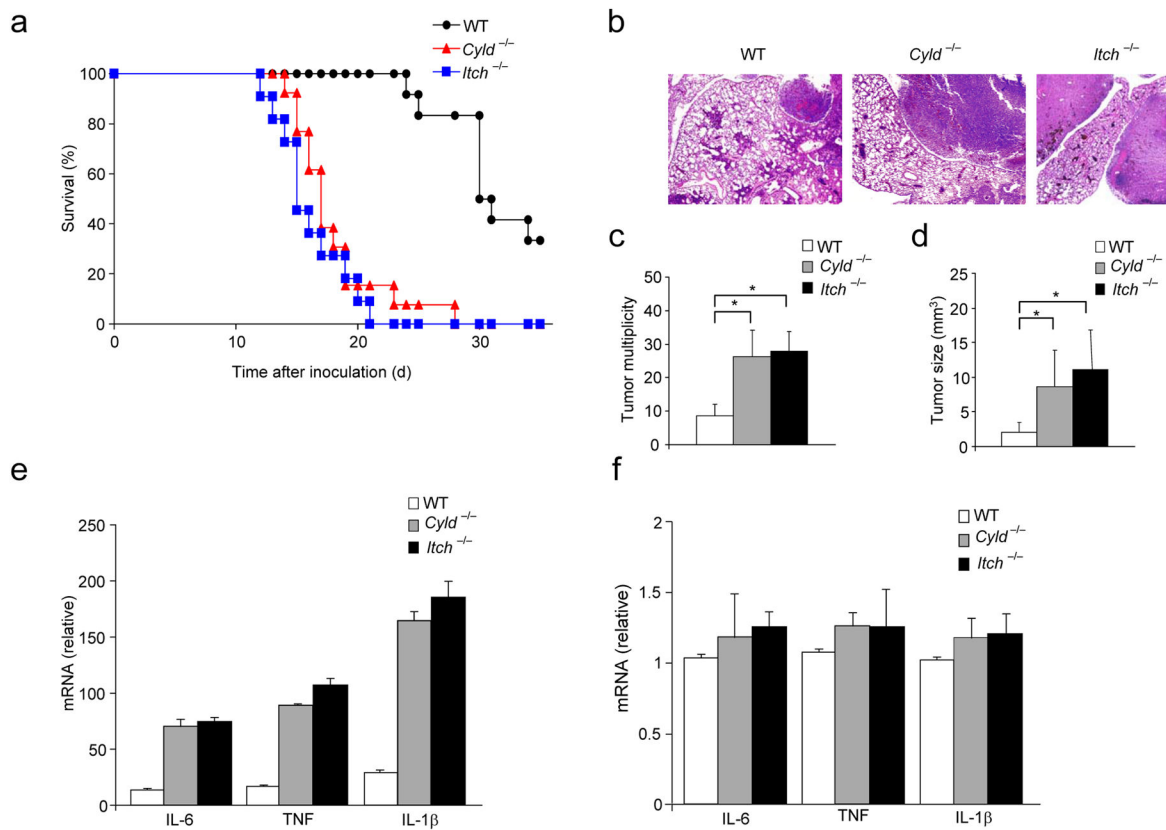
We thank William C. Hahn (Dana-Farber Cancer Institute, Boston, MA) for Myc-Cyld, Jonathan Ashwell (NCI, Bethesda, MD) for HA tagged TRAF2, Avraham Raz for pET30a, Yi-Chi M. Kong and Ramkumar Mathur (Columbia University, NY), for helpful discussions. This work was supported by National Cancer Institute (NCI) grant 1RC1CA146576-01 to K.V.

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**Figure 1.**

Itch^{-/-} and *Cyld*^{-/-} mice develop elevated growth and metastasis of Lewis Lung Carcinoma.

(a) % survival of WT (n=12), *Cyld*^{-/-} (n=13) and *Itch*^{-/-} (n=11) mice inoculated with 1×10^6 Lewis Lung Carcinoma (LLC) cells via tail vein. (b) Representative H&E stained histological sections of the lungs of wild type, *Itch*^{-/-} and *Cyld*^{-/-} mice on day 15 after inoculation of LLC cells (1×10^6 cells). (c) Lung tumor multiplicity and (d) Tumor size. (e) Cytokine mRNA levels in CD11b⁺ macrophages from lung tumors of WT, *Itch*^{-/-} and *Cyld*^{-/-} mice inoculated with LLC cells (1×10^6) via tail vein. (f) Cytokine abundance in naïve *Itch*^{-/-} and *Cyld*^{-/-} lung tissues. *P<0.01 (paired t-test). Data are representative of three independent experiments.

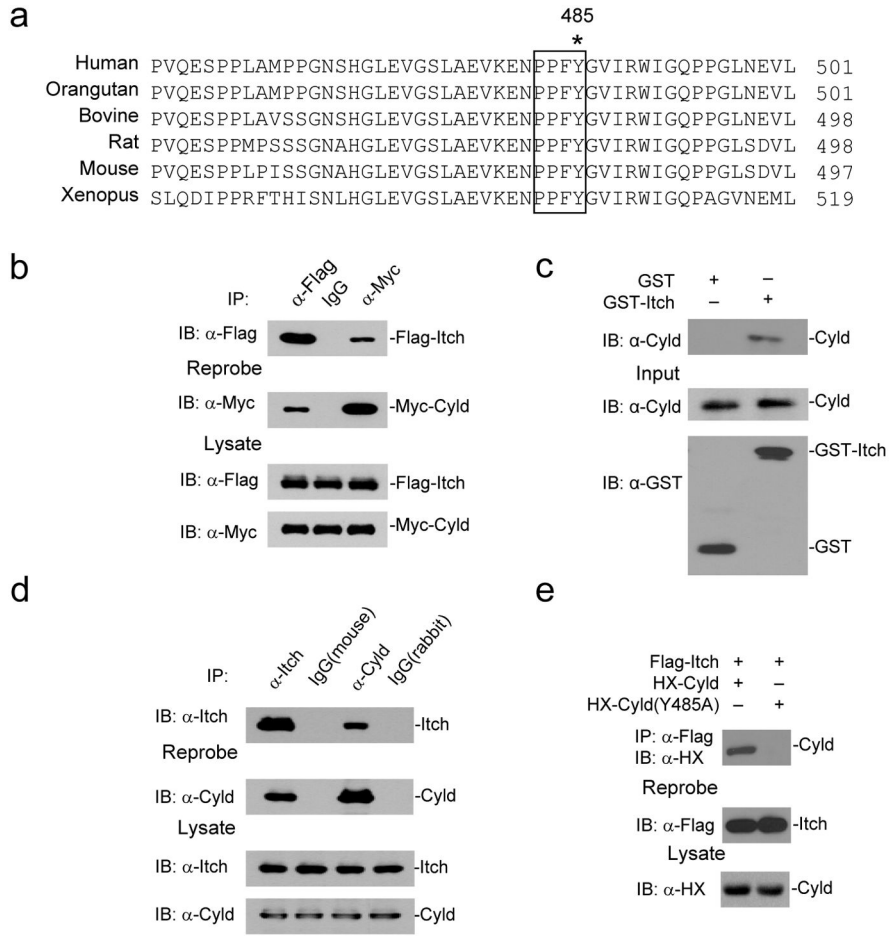
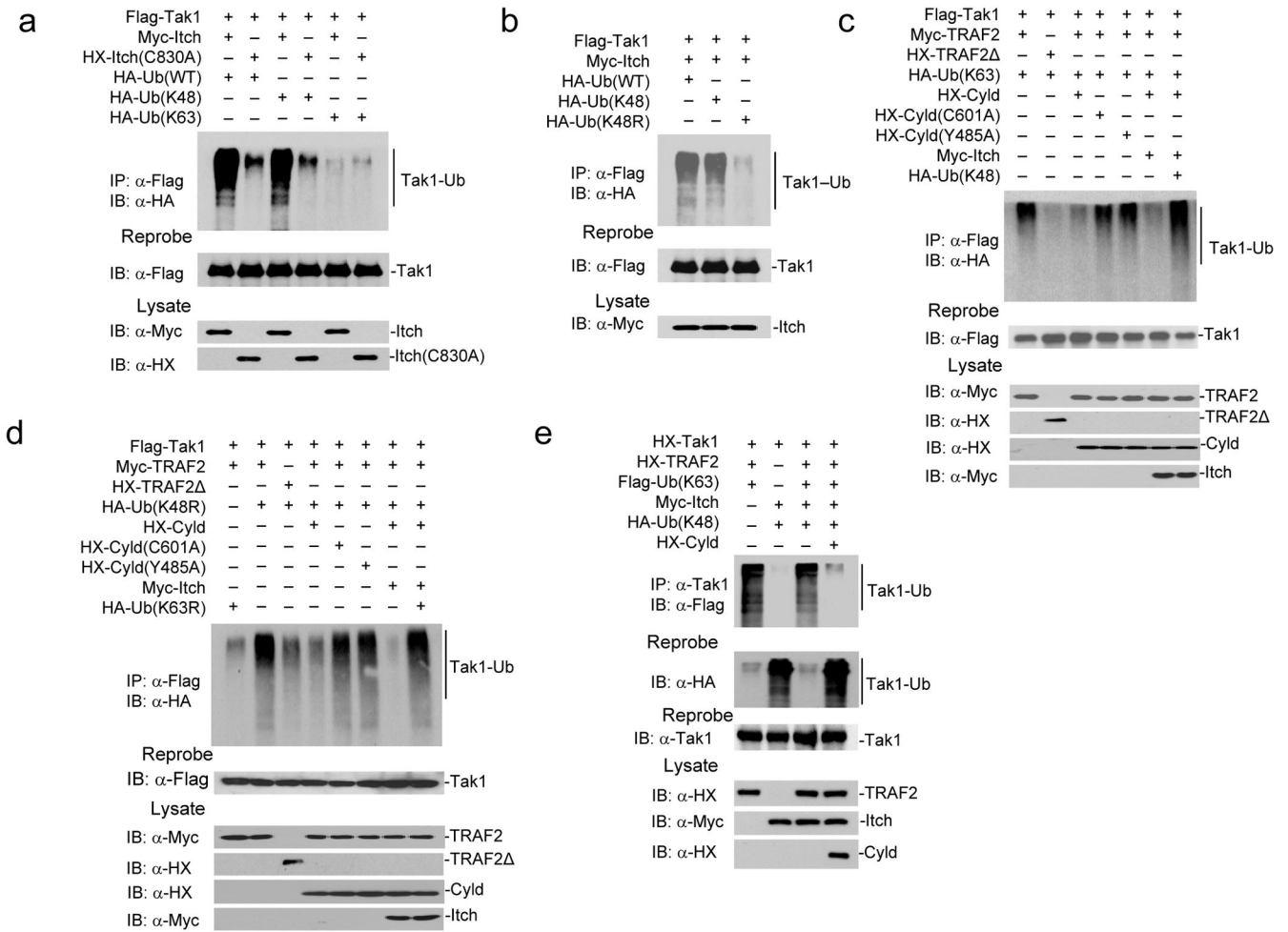


Figure 2. Itch and Cyld form a complex *via* interaction through PPXY motif. **(a)** Sequence alignment of PPXY motif of Cyld which is conserved across species. Y485 critical for interaction with Itch is indicated (*). **(b)** 293T cells transiently co-transfected with expression vectors for Flag-Itch and Myc-Cyld. The cell lysate was immunoprecipitated using mouse IgG, antibodies against Flag and Myc. The immunoprecipitates were blotted using antibodies against Flag and Myc. **(c)** Immunoblot of His-Cyld precipitated using GST or GST-Itch purified from *E. coli*. The input controls for His-Cyld, GST and GST-Itch are shown below **(d)** BMDMs were stimulated with TNF for thirty minutes. The cell lysates were immunoprecipitated using mouse IgG, rabbit IgG, antibodies against Itch and Cyld. The immunoprecipitates were analyzed by immunoblot with antibodies against Itch and Cyld. **(e)** 293T cells were co-transfected with expression vectors for HX-Cyld(Y485A) and Flag-Itch. The cells lysate was immunoprecipitated using antibody against Flag and immunoblotted using antibody against HX. Data are representative of three **(b, c and e)** and two **(d)** experiments.

**Figure 3.**

Itch and Cyld sequentially cleave K63-linked ubiquitination and catalyze K48-linked ubiquitination to deactivate Tak1. **(a)** 293T cells co-transfected with Flag-Tak1 along with Myc-Itch, HX-Itch (C830A), HA tagged Ub(WT), Ub(K63) and Ub(K48) as indicated. The cell lysate was immunoprecipitated using antibody against Flag and immunoblotted using antibody against HA. **(b)** 293T cells co-transfected with Flag-Tak1 along with Myc-Itch, HA-Ub(WT), HA-Ub(K48) and Ub(K48R) as indicated. The cell lysate was immunoprecipitated using antibody against Flag and immunoblotted using antibody against HA. **(c)** 293T cells co-transfected with Flag-Tak1 along with Myc-TRAF2, HX-TRAF2, HA-Ub(K63), HX-Cyld, HX-Cyld(C601A), HX-Cyld(Y485A), Myc-Itch, HA-Ub(K48) in various combinations as indicated. The cell lysate was immunoprecipitated using antibody against Flag and immunoblotted using antibody against HA. **(d)** 293T cells were transfected as in **(b)** utilizing Ub(K48R) and Ub(K63R). **(e)** HX-Tak1, HX-TRAF2 and Flag-Ub(K63), HA-Ub(K48), Myc-Itch, and HX-Cyld were co-expressed in 293T cells. The cell lysate was immunoprecipitated using antibody against Tak1 and immunoblotted with antibody against Flag to detect K63-linked ubiquitination. The membranes were reprobed with antibody against HA to detect K48-linked ubiquitination. Data are representative of three or more independent experiments.

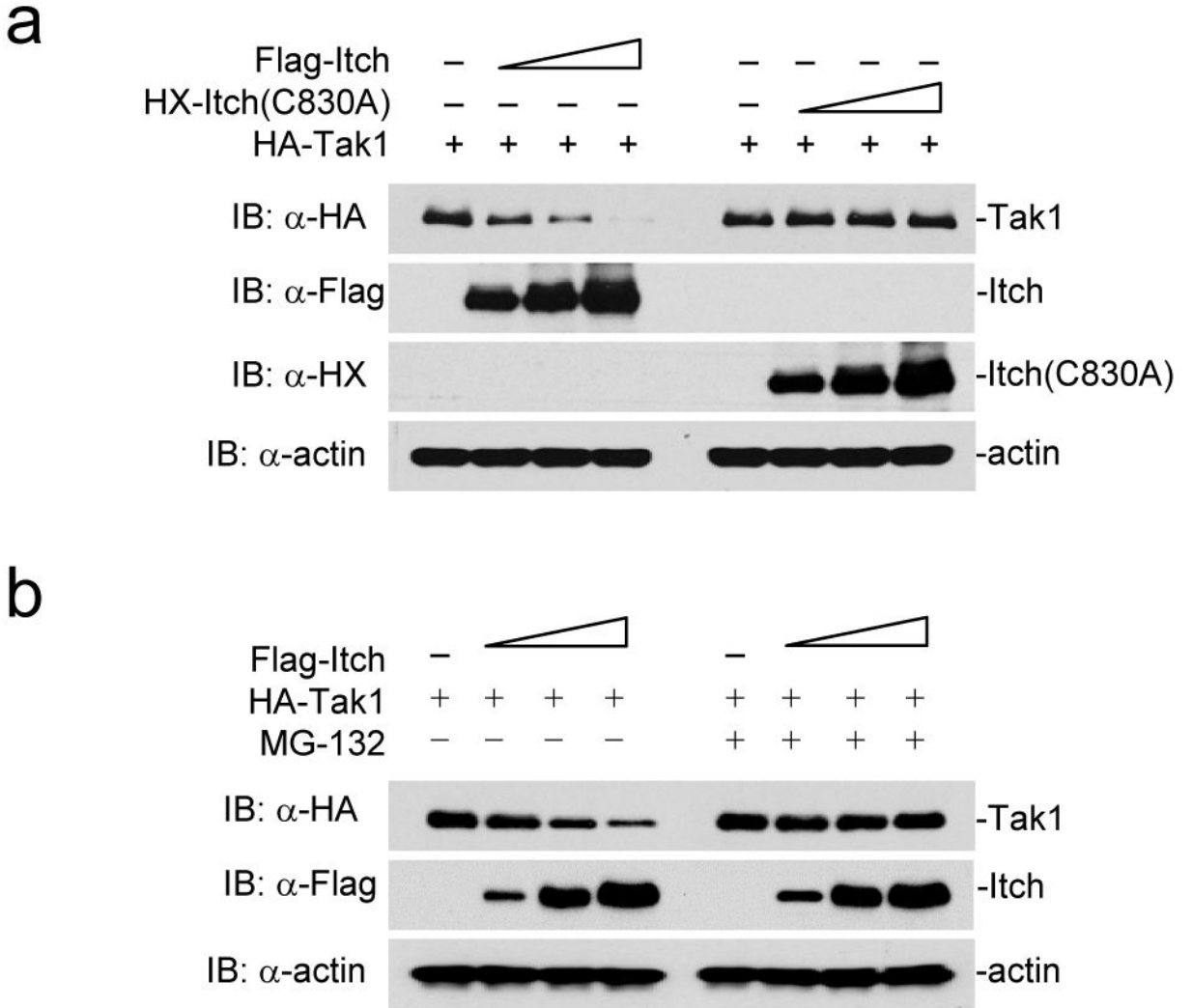


Figure 4. Itch-mediated polyubiquitination results in Tak1 degradation. **(a)** 293T cells were co-transfected with HA-Tak1 (1 μ g) and increasing concentrations of Flag-Itch or Itch(C830A). The cellular Tak1 level was analyzed by immunoblotting the lysate using antibody against HA. The level of expression of Itch and Itch-C830A was analyzed by reprobating the membranes using antibodies against Flag and HX. **(b)** 293T cells were transfected with HA-Tak1 (1 μ g) and increasing concentrations of Flag-Itch, the cells were cultured in the presence or absence of MG132. The cellular Tak1 level was analyzed by immunoblotting the lysate using antibody against HA. Data are representative of two experiments.

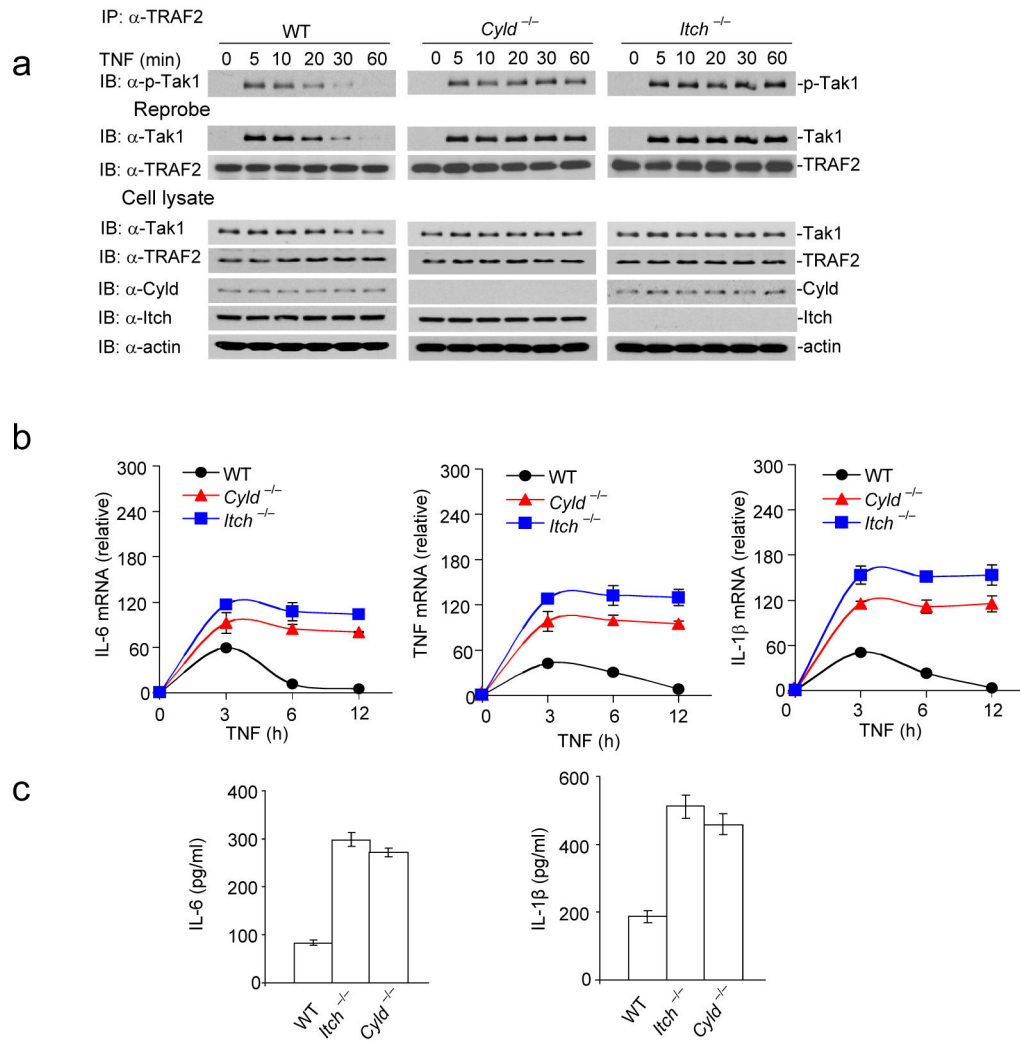
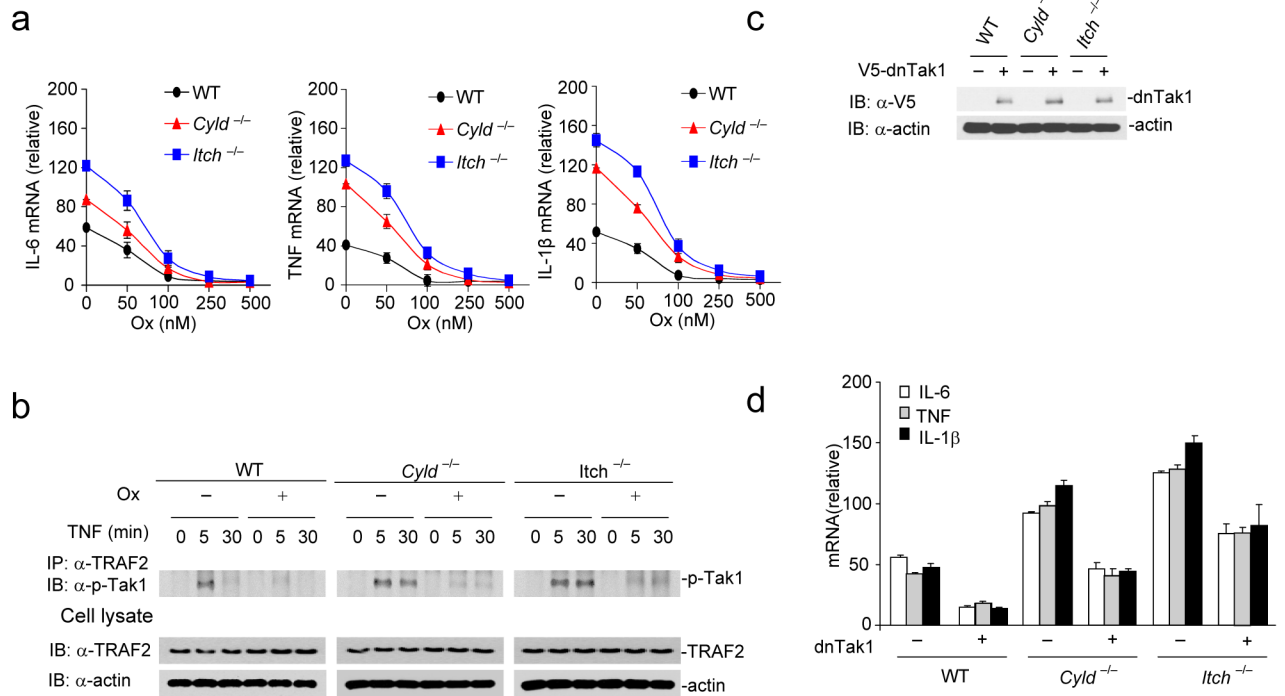
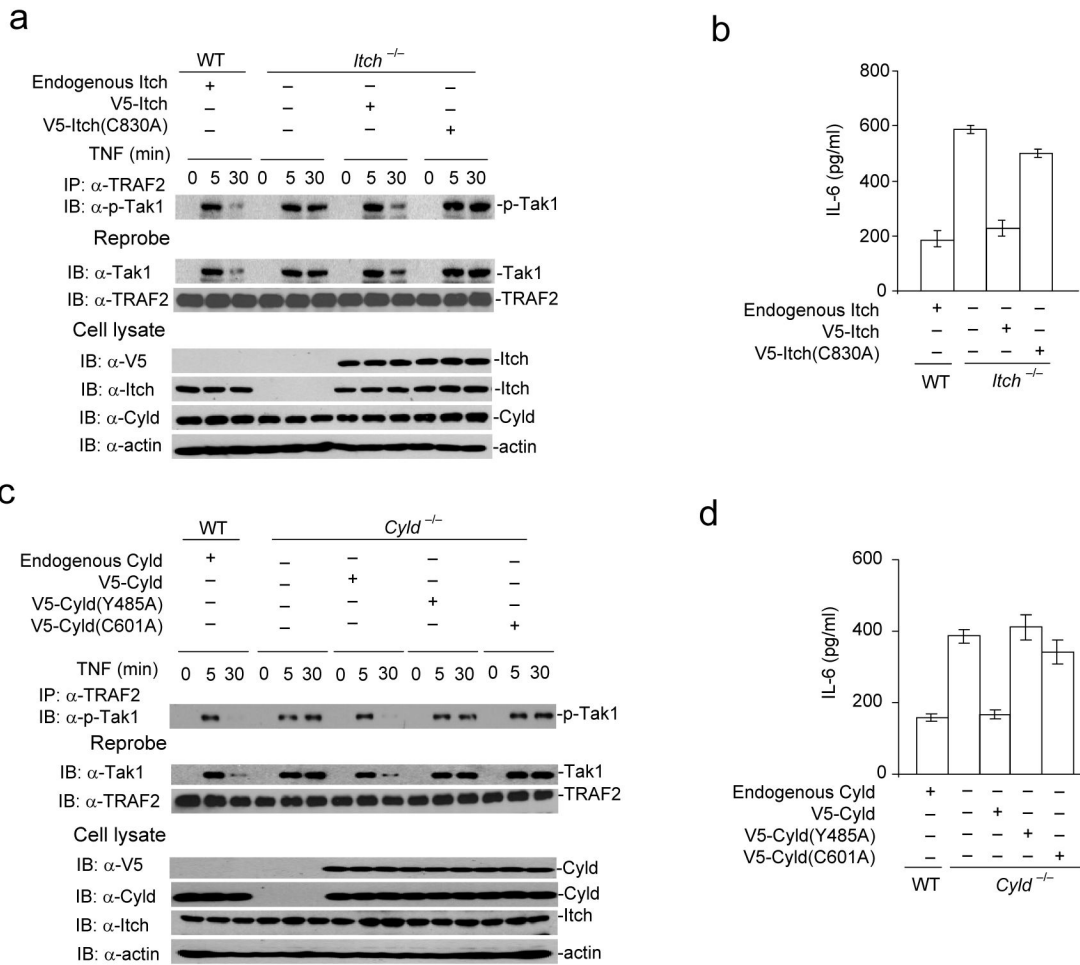


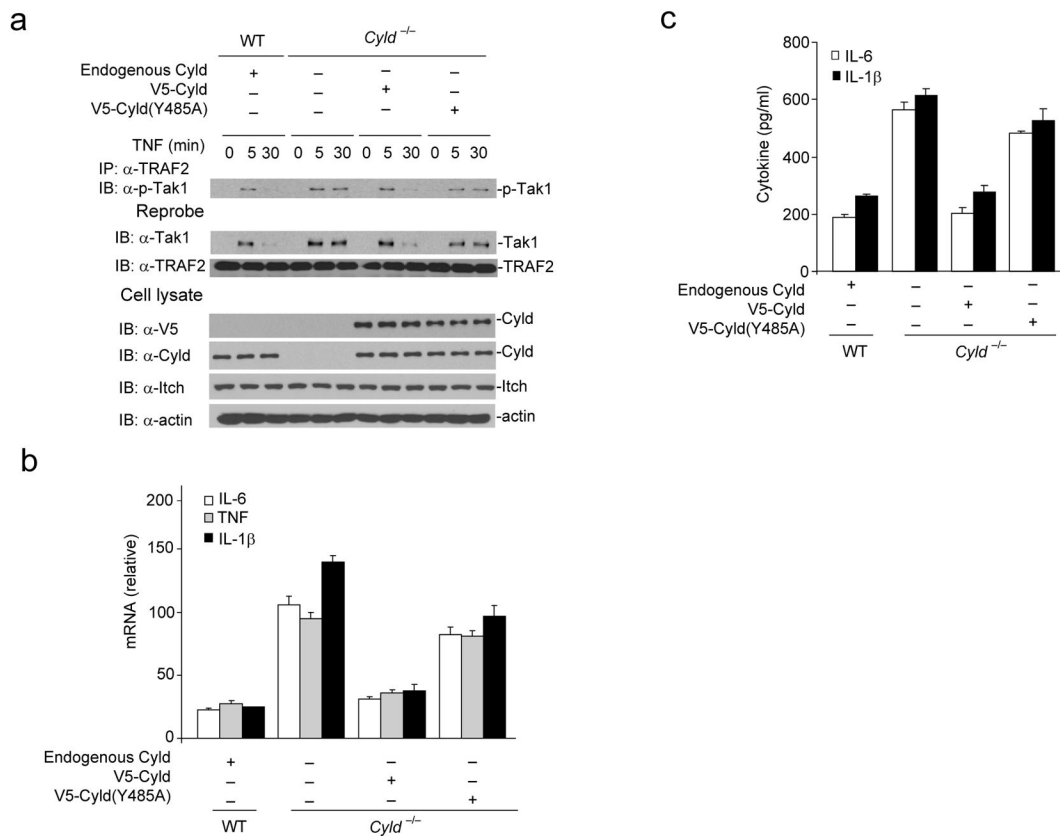
Figure 5. Sustained Tak1 activation results in chronic production of inflammatory cytokines by *Itch*^{-/-} and *Cyld*^{-/-} BMDMs. **(a)** *Itch*^{-/-} and *Cyld*^{-/-} BMDMs were stimulated with TNF and lysed at the indicated time points. The lysate was immunoprecipitated with antibody against TRAF2 and immunoblotted with antibody against p-Tak1. The membranes were reprobed with antibodies against Tak1 and TRAF2. The total cell lysate was blotted with antibodies against Tak1, TRAF2, Cyld, Itch and actin. **(b)** BMDMs were stimulated with TNF, total RNA was isolated at the indicated time points and expression of IL-6, TNF and IL-1β was analyzed using real-time PCR. **(c)** IL-6 and IL-1β concentration were assayed in the culture supernatant by ELISA. Data are representative of three experiments (**b–c** mean and s.d. of triplicate wells).

**Figure 6.**

Inhibiting Tak1 activation rescues elevated inflammatory cytokine production by *Itch*^{-/-} and *Cyld*^{-/-} BMDMs **(a)** BMDMs were pre-incubated with indicated amounts of (5Z)-7-Oxozeaenol(Ox) and stimulated with TNF for 6 hours, expression of IL-6, TNF, and IL-1 β was analyzed by real-time PCR. **(b)** BMDMs were pretreated with 100 nM of Ox for 30 minutes and then stimulated with TNF for the indicated time points. The cell lysates were immunoprecipitated using antibody against TRAF2 and immunoblotted using antibody against p-Tak1. The total lysate was immunoblotted using antibodies against TRAF2 and actin. **(c)** WT, *Itch*^{-/-} and *Cyld*^{-/-} BMDMs were transduced with a lentiviral vector encoding dominant negative Tak1 (dnTak1). The efficiency of ectopic expression was analyzed by immunoblotting the total cell lysate using antibody against V5. **(d)** WT, *Itch*^{-/-} and *Cyld*^{-/-} BMDMs transduced with dnTak1 were stimulated with TNF and expression of IL-6, TNF and IL-1 β was analyzed by real-time PCR. Data are representative of three experiments. **(a, d)** mean and s.d. of triplicate wells).

**Figure 7.**

Ligase activity of Itch, DUB activity of Cyld, and Cyld-Itch interaction is necessary for termination of TNF induced Tak1 activation. **(a)** *Itch*^{-/-} MEFs were reconstituted with either V5-Itch(WT) or V5-Itch(C830A) mutant using a lentiviral transduction system. The cells lysed at indicated time points post TNF stimulation. The lysate was immunoprecipitated using against TRAF2 and immunoblotted with antibody against p-Tak1. The same membrane was reprobed with antibody against Tak1. The cell lysate from WT MEFs stimulated with TNF was used as control. **(b)** *Itch*^{-/-} MEFs transduced with V5-Itch(WT) or V5-Itch(C830A) mutant as in **(a)** were stimulated with TNF and expression of IL-6 was analyzed by ELISA in the culture supernatant. **(c)** *Cyld*^{-/-} MEFs were reconstituted with V5-Cyld(WT), V5-Cyld(C601A) or V5-Cyld(Y485A) mutant. The cells lysed at indicated time points post TNF stimulation. The lysate was immunoprecipitated using antibody against TRAF2 and immunoblotted with antibody against p-Tak1. The same membrane was reprobed with antibody against Tak1. **(d)** *Cyld*^{-/-} MEFs transduced with V5-Cyld(WT), V5-Cyld(Y485A) or V5-Cyld(C601A) mutant as in **(c)** stimulated with TNF and expression of IL-6 was analyzed by ELISA in the culture supernatant. Data are representative of three **(b, d)** and two **(a, c)** experiments. **(b, d)** mean and s.d. of triplicate wells).

**Figure 8.**

Reconstitution of *Cyld*^{-/-} BMDMs with WT but not *Cyld*(Y485A) mutant rescues defects in termination of Tak1 activation and chronic production of inflammatory cytokines. **(a)** V5-*Cyld*(WT) or V5-*Cyld*(Y485A) mutant was ectopically expressed in *Cyld*^{-/-} BMDMs using a lentiviral transduction system. The cells lysed at indicated time points post TNF stimulation. The lysate was immunoprecipitated using antibody against TRAF2 and immunoblotted with antibody against p-Tak1. The same membrane was reprobed with antibody against Tak1. The cell lysate from WT BMDMs stimulated with TNF was used as control in these experiments. **(b)** WT and *Cyld*^{-/-} transduced with V5-*Cyld*(WT) or V5-*Cyld*(Y485A) mutant were stimulated with TNF and expression of IL-6, TNF and IL-1β was analyzed by real-time PCR. **(c)** IL-6 and IL-1β concentration were assayed in the culture supernatant by ELISA. Data are representative of at least three independent experiments (**b–c** mean and s.d. of triplicate wells).