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Cylindromatosis (CYLD) inhibits *Streptococcus pneumonia*-induced plasminogen activator inhibitor-1 expression via interacting with TRAF-6

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ABSTRACT

Streptococcus pneumoniae (*S. p*) remains one of the foremost causes of community-acquired pneumonia. Recent studies have shown that *S. p lung infection* is associated with plasminogen activator inhibitor-1 (PAI-1) expression, which inhibits acute lung injury. Such effects by *S. p* were negatively regulated by cylindromatosis (CYLD). The current study explored the underlying mechanisms. We showed that *S. p*-induced PAI-1 expression requires tumor necrosis factor receptor-associated factor 6 (TRAF-6) signaling. Si-RNA-mediated knockdown of TRAF-6 remarkably inhibited *S. p*-induced PAI-1 expression. Reversely, over-expression of wild type (wt-) TRAF-6 further potentiated PAI-1 expression in *S. p*-treated cells. We provided evidences to support that CYLD-mediated anti-PAI-1 activity might be through direct regulation of TRAF-6. Our results from co-immunoprecipitation (co-IP) and confocal microscopy assays confirmed a direct association between the CYLD and TRAF-6 in A549 cells. Over-expression of wt-CYLD remarkably inhibited TRAF-6 ubiquitination and subsequent PAI-1 expression. Introducing a mutated CYLD, on the other hand, enhanced TRAF-6 ubiquitination and PAI-1 expression. Together, these results indicate that TRAF-6 mediates *S. p*-induced PAI-1 expression, and CYLD inhibits PAI-1 expression probably through deubiquitinating TRAF-6. The current study provided molecular insights of CYLD-mediated activities in *S. p*-induced PAI-1 expression and possible acute lung injury.

1. Introduction

Streptococcus pneumoniae (*S. p*), a gram-positive pathogenic bacterium, remains one of the foremost causes of community-acquired pneumonia [1–3]. Meanwhile, *S. p* infections could also cause other diseases, *i.e.* bronchitis and meningitis [1–3]. *S. p*-induced inflammatory responses in lung are often not well controlled, which will likely lead to acute lung injury [4]. Thrombosis and fibrinolysis are often found in sepsis patients with *S. p* infections [5]. Thus, groups all over the world are studying the underlying mechanisms of *S. p*-induced inflammatory responses, and to explore possible intervention strategies [6,7].

Plasminogen activator inhibitor-1 (PAI-1) is an important fibrinolysis inhibitor [8]. Expression of PAI-1 is often increased after inflammation, which inhibits fibrosis and protects tissues from subsequent injuries [9,10]. Existing evidences have identified PAI-1 as an essential component of pulmonary host defense during *Pseudomonas aeruginosa* pneumonia [11]. The PAI-1 level in bronchoalveolar lavage fluid of *S. p* pneumonia rats was significantly higher than that of healthy animals [12]. These studies implicated a possible role of PAI-1 in *S. p*-induced inflammatory responses and tissues damages.

The deubiquitinating enzyme cylindromatosis (CYLD) was first identified as a tumor suppressor mutated in familial cylindromatosis [13]. Later on, studies have confirmed it's negative regulatory role for several key inflammatory signalings [14] [13,15–17]. CYLD could be induced by bacterial pathogens, and directly interacts with various immune signaling molecule, including tumor necrosis factor receptor (TNF)–associated factor 6 (TRAF-6) [17], TRAF2 and NEMO (NF-kB essential modulator), among others [13,15,16]. Studies have showed that CYLD works as a deubiquitinase to remove lysine 63-linked poly-ubiquitin chain from targeted proteins [13,15,16]. For example, CYLD directly

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associates and deubiquitinates NEMO and TRAF-6, thus inhibiting TNF-α-induced nuclear factor-κB (NF-κB) signaling [13,15–17].

A previous study has demonstrated that CYLD deficiency protected *S. p*-induced acute lung injury and lethality in mice [18]. The authors showed that CYLD inhibited *S. p*-induced PAI-1 expression in lung, thereby potentiating pulmonary damages [18]. The underlying mechanisms are not fully understood. In this study, we provided evidence to show that CYLD inhibits PAI-1 expression probably through directly interacting with and deubiquitinating TRAF-6.

2. Materials and methods

2.1. Bacteria and lysates

Clinical isolations of *S. p* strain (serotype 2) were utilized in our study. *S. p* was grown on chocolate agar plates and transferred to Todd–Hewitt broth supplemented with 0.5% yeast extract at 37 °C in the humidified incubator with 5% CO_2. *S. p* lysates were prepared as previously described [19]. The lysates were stored at −80 °C, and 5 μg/mL lysates were utilized in all experiments. The study was approved by the institutional review board. All investigations regarding clinical samples were conducted according to the principles expressed in the Declaration of Helsinki.

2.2. Cells, plasmids, siRNAs and transfection

A549, HeLa and THP-1 cells, all purchased from the FuDan IBS Cell Center (Shanghai, China), were maintained in the F12-K or RPMI-1640 medium containing 10% fetal bovine serum (FBS, Gibco, Shanghai, China). The expression plasmids of wild type (wt-) TRAF-6, Flag-wt-CYLD, enzymatically-inactive mutant of CYLD (CYLD H/N), catalytically-inactive mutant of CYLD (CYLD C/S) and PAI-1-Luc reporter were gifts from Prof. Li's lab (Georgia State University) [20]. Basic plasmid pcDNA was utilized as the empty vector. TRAF-6 siRNA and the scramble control siRNA were purchased from Qiagen (Hilden, Germany). Cells were transiently transfected with applied plasmid or siRNA using TransIT-LT1 reagent (Mirus Bio, Madison, WI) or Lipofectamine 2000 (Invitrogen Life Technologies, Shanghai, China) according to manufacturers' instructions.

2.3. qRT-PCR analysis

Total RNA was extracted through TRIzol reagent (Invitrogen Life Technologies, Shanghai, China), according to manufacturer's instructions. cDNA was transcribed using the Transcriptor First Strand cDNA Synthesis Kit (Takara Bio, Kyoto, Japan). Quantitative real-time PCR was performed using a SYBR Green PCR kit (Takara Bio) on ABI 7500 platform (Applied Biosystems, Connecticut Path, Framingham). After amplification, melt curve analysis was performed to analyze product melting temperature. Glyceraldehydes-3-phosphate dehydrogenase (GAPDH) gene was chosen as the reference gene for normalization, and the $2^{-\Delta\Delta Ct}$ method was applied to quantify targeted mRNA change within samples [21]. The primers for PAI-1 were previously described [22]. The primers for TRAF-6 were 5′-CCTTTGGCAAATGTCATCTGTG-3' (forward) and 5′-CTCTGCATCTTTTCATGGCAAC-3' (reverse).

2.4. Luciferase reporter assay

In reporter assay, A549 cells cultured in 12-well plates were grown to 60%–70% confluence, and then transfected with 100 ng of PAI-1-Luc plasmid/well with TransIT-LT1 reagent. After transfection (24 h), cells were treated with *S. p* lysates or PBS for indicated time. Cell lysates were harvested, and the reporter activity was measured with the luciferase assay kit (Promega, Shanghai, China) following manufacturer's instructions. Transforming growth factor-beta (TGF-β), utilized as a positive control, was purchased from Sigma.

2.5. Western blots and co-immunoprecipitation (IP)

The antibodies utilized in this study were purchased from Cell Signaling Technology (Shanghai, China). Western blot analyses were performed as previously described [23]. Cells were treated with *S. p* lysates for indicated time. Whole-cell lysates were separated in a 8%–10% SDS-PAGE gel, and were transferred to polyvinylidene fluoride (PVDF) membrane, which was incubated with indicated primary antibody and corresponding second antibody. Targeted proteins were visualized by the ECL detection system (GE Healthcare, Shanghai, China). For Co-IP assay, aliquots of 600 μg of protein lysates from each sample (in 1 mL of lysis buffer) were pre-cleared by incubation with 30 μl of protein A/G Sepharose (beads) (Sigma) for 1 h at 4 °C. The pre-cleared samples were incubated with the specific primary antibody (anti-TRAF-6, 2 μg/mL) in lysis buffer overnight at 4 °C. 30 μl of protein A/G beads were added, and the samples were incubated for 2 h at 4 °C. The beads were washed five times with PBS (4 °C) and once with lysis buffer, boiled, separated by 10% SDS-PAGE, and transferred onto a PVDF membrane followed by Western blot analysis.

2.6. Immunocytochemical staining

For Immunocytochemical staining, A549 cells were cultured on 10-mm cover-slips. Cells were then transfected with wt-TRAF-6 and FLAG-wt-CYLD plasmid with Lipofectamine 2000 reagent (Invitrogen, Shanghai, China). 24 h after transfection, cells were fixed by 4% paraformaldehyde, and treated with 0.5% Triton X-100 at room temperature for 30 min, followed by 10% normal goat serum incubation. Afterwards, rabbit anti-human anti-TRAF-6 antibody (1:200 dilution) and mouse anti-human anti-FLAG antibody (1:200 dilution) were added. The cells were stained for 1 h with fluorescein isothiocyanate-conjugated anti-mouse secondary antibody (Santa Cruz Biotech) and Cy3-conjugated anti-rabbit secondary antibody (Santa Cruz Biotech). The nuclei were stained with DAPI (Invitrogen). CYLD (green fluorescence) and TRAF-6 (red fluorescence) co-localization was viewed under a confocal fluorescence microscope (Ni–U, Nikon, Shanghai, China).

2.7. Statistical analysis

All experiments were repeated in at least three times, and similar results were obtained. Data presented were expressed as means ± standard deviation (SD). Statistical differences were analyzed by one-way *ANOVA* followed by multiple comparisons performed with post hoc Bonferroni test (SPSS version 18.0, Chicago, IL). $P < 0.05$ was considered statistically significant.

3. Results

3.1. S. p lysates up-regulates PAI-1 expression in multiple cell lines with different background

First, we tested the effect of *S. p* on PAI-1 expression through qRT-PCR assay. As shown in Fig. 1A and Fig. 1B, PAI-1 mRNA expression was significantly increased after treatment of *S. p* lysates in both A549 lung adenocarcinoma epithelial cells, and in THP-1 monocyte-derived macrophages. Further, luciferase assay results demonstrated that the PAI-1 promoter activity was also increased by the applied *S. p* stimulation (2 h after *S. p* treatment, Fig. 1C). TGF-β,

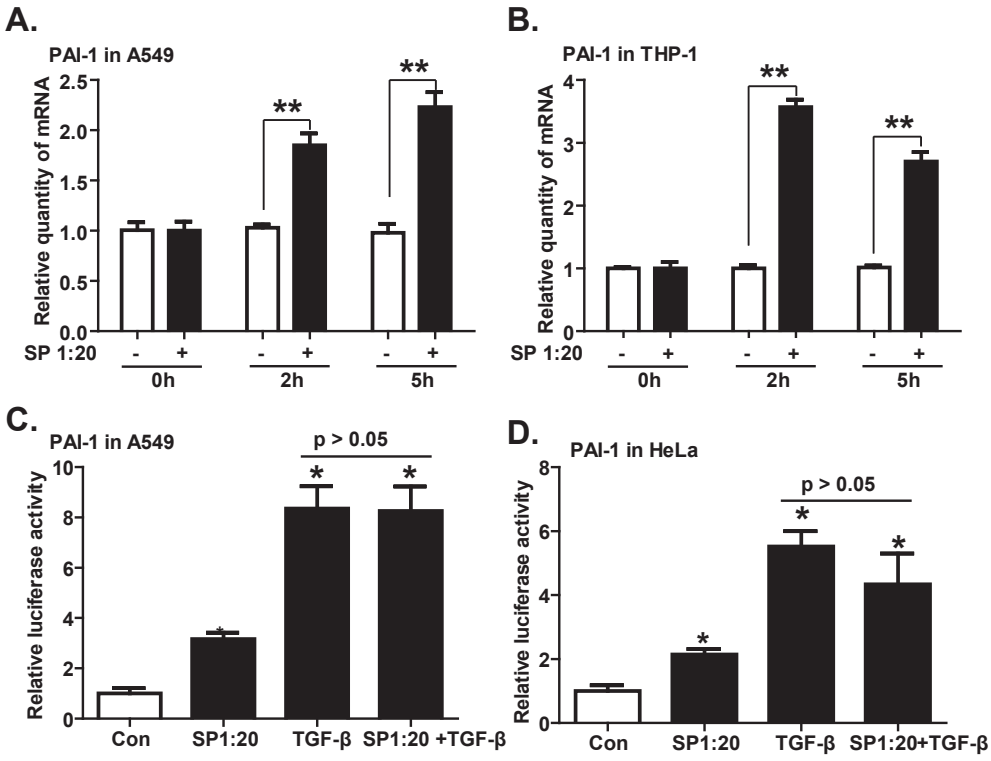

Fig. 1. *S. p* induces PAI-1 expression in multiple cell lines. The relative PAI-1 mRNA level (vs. 0 h) in A549 cells (A) and THP-1 cells (B) before and after indicated *S. p* lysates treatment. The relative PAI-1 promoter activity (vs. "Con") in A549 cells (C) and HeLa cells (D) before and after indicated *S. p* lysates or plus TGF-β (25 ng/mL) treatment. Data were shown as mean ± SD ($n = 5$) of one representative experiment. Similar results were obtained from three independent experiments. "Con" stands for medium control. $^{**}P < 0.05$. $^{*}P < 0.05$ vs. "Con" group (C and D).

served as a positive control, also induced PAI-1 promoter activity increase (Fig. 1C) [24]. Interestingly, there was no additive effect between TGF-β and *S. p* lysates in promoting PAI-1 expression in A549 cells (Fig. 1C). Above results were also observed in HeLa cells (Fig. 1D). Based on these results, we show that *S. p* up-regulates PAI-1 expression in multiple cell lines with different background.

3.2. CYLD's inhibition on S. p-induced PAI-1 expression through regulating TRAF-6

Above results demonstrated that *S. p* up-regulates PAI-1 expression. Next, we assessed the effect of CYLD on PAI-1 expression. Wild-type CYLD (wt-CYLD) [20] was transfected into A549 cells. As shown in Fig. 2A, over-expression of wt-CYLD remarkably inhibited *S. p*-induced PAI-1 expression, and the effect of CYLD was dose dependent (Fig. 2A). On the other hand, over-expression of an enzymatically-inactive mutant of CYLD (CYLD H/N) [20] or a catalytically-inactive mutant of CYLD (CYLD C/S) [20] potentiated *S. p*-induced PAI-1 expression (Fig. 2B). Note that the plasmids utilized in this study were from Prof. Li's lab at Georgia State University [20], in pre-experiments, real-time PCR assay was performed to verify these constructs. These results confirm that CYLD negatively regulates *S. p*-mediated PAI-1 expression.

As discussed early, CYLD exerted its activity on several inflammatory signalings through deubiquitinating targeted kinases, including TRAF-6 [17]. We then investigated the potential role of TRAF-6 on *S. p*-mediated PAI-1 expression. The DNA construct expressing wt-TRAF-6 was transfected into A549 cells. Without *S. p* treatment, over-expression of wt-TRAF-6 failed to affect PAI-1 expression (Fig. 2C). Remarkably, *S. p*-induced PAI-1 expression was enhanced in TRAF-6-overexpressed A549 cells (Fig. 2C). On the other hand, siRNA-mediated knockdown of TRAF-6 inhibited PAI-1 expression in *S. p*-infected A549 cells (Fig. 2D). Similar results were also obtained in THP-1 cells (Supplementary Fig. 1A) and in HeLa cells (Supplementary Fig. 1B), where TRAF-6 was again important for *S. p*-induced PAI-1 expression. The efficiency of siRNA in reducing endogenous TRAF-6 expression was confirmed by real-time PCR assay (Fig. 2D, up panel). More importantly, we showed that TRAF-6-mediated over-expression of PAI-1 was inhibited by co-expression of wt-CYLD in *S. p*-infected A549 cells, suggesting that CYLD may target TRAF-6 in inhibiting PAI-1 expression (Fig. 2D). These wt-CYLD and wt-TFAF6 co-expression experiments were also repeated in THP-1 and HeLa cells, and similar results were observed (Supplementary Fig. 1A and 1B).

3.3. CYLD physically interacts with TRAF-6 in A549 cells

Above results have shown that CYLD inhibits *S. p*-induced PAI-1 expression possibly through regulating TRAF-6. However, it is unknown whether there is a direct interaction between CYLD and TRAF-6, and if so, how CYLD regulates TRAF signaling. To answer this question, we co-transfected Flag-CYLD plasmid and wt-TRAF-6 plasmid into A-549 cells, and co-immunoprecipitation (co-IP) assay was performed. Results in Fig. 3A demonstrated that TRAF-6 formed a complex with wt-CYLD in A549 cells. Confocal microscopy assay results further confirmed CYLD-TRAF-6 co-localization in A549 cells (Fig. 3B). These results show that CYLD is indeed physically associated with TRAF-6.

3.4. CYLD deubiquitinates TRAF-6 in A549 cells

Next, we explored the physical relevance of this TRAF-6-CYLD interaction. Above results have shown that TRAF-6 is required for *S. p*-induced PAI-1 expression. It is known that activation of TRAF-6

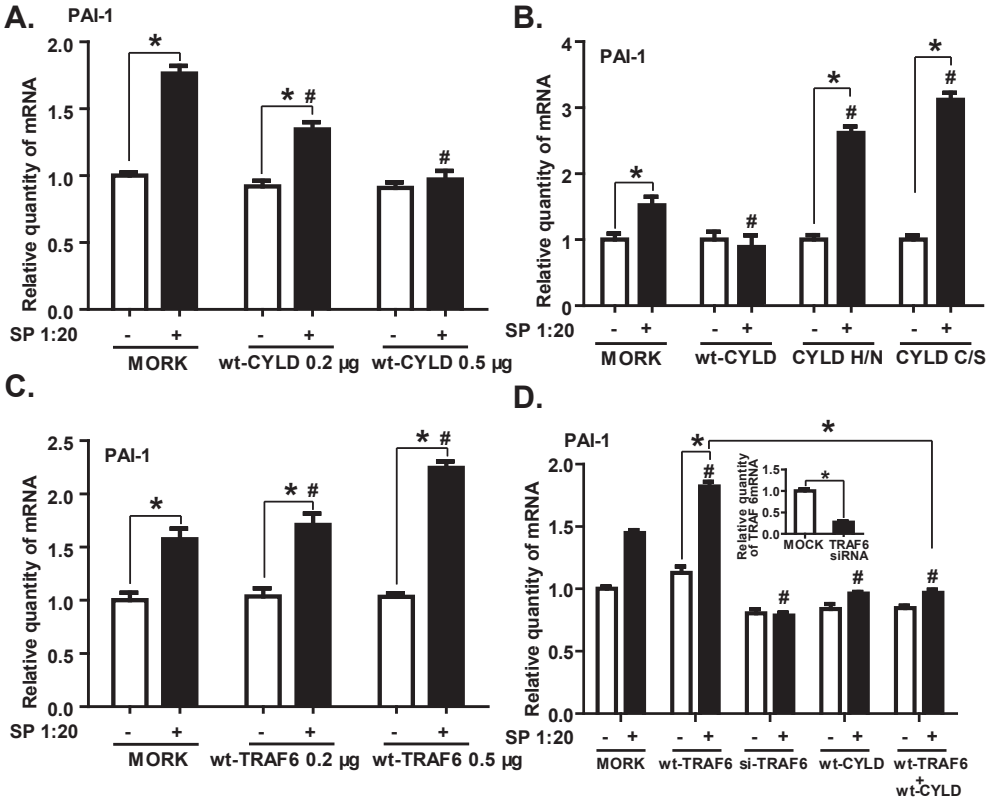

Fig. 2. CYLD inhibits *S. p*-induced PAI-1 expression in A549 cells. A549 cells, transfected with empty vector ("MORK") or indicated plasmid, wild type (wt-) CYLD, the enzymatically-inactive mutant of CYLD (CYLD H/N), catalytically-inactive mutant of CYLD (CYLD C/S) or wt-TRAF-6, were treated with or without *S. p* lysates for 5 h, relative PAI-1 mRNA expression was shown (A–C). A549 cells, transfected with empty vector ("MORK"), wt-TRAF-6 cDNA, TRAF-6 siRNA, wt-CYLD cDNA or wt-TRAF-6 cDNA + wt-CYLD cDNA, were treated with *S. p* lysates for 5 h, relative PAI-1 mRNA expression was shown (D). Relative expression of TRAF-6 mRNA (vs. MORK group) in MORK- or TRAF-6 siRNA-transfected A549 cells was shown (D, upper panel). Data were shown as mean ± SD ($n = 5$) of one representative experiment. Similar results were obtained from three independent experiments. $^{*}P < 0.05$. $^{\#}P < 0.05$ vs. *S. p* treatment of MORK group.

requires Lys-63-linked poly-ubiquitination, and CYLD is a well-known deubiquitinase [13,15,16,25,26]. Thus, we tested the possible effect of CYLD on *S. p*-induced TRAF-6 ubiquitination in A-549 cells. Again, wt-TRAF-6 and/or CYLD were transfected into A549 cells, *S. p* lysates induced significant TRAF-6 ubiquitination, which was remarkably inhibited by co-expression of wt-CYLD (Fig. 3C). On the other hand, over-expression of the H/N mutant CYLD potentiated TRAF-6 ubiquitination (Fig. 3C). Based on these results, we propose the following model: *S. p*-induced PAI-1 expression requires TRAF-6 ubiquitination and activation. CYLD forms a complex and deubiquitinates TRAF-6, thus in-activating TRAF-6, and inhibiting subsequent PAI-1 expression (See Fig. 4).

4. Discussion

PAI-1 plays an important role in preventing excessive hemorrhage and tissue injuries [27,28]. It can be induced by bacterial infection of *Staphylococcus aureus*, *P. aeruginosa* and *S. p* [27,28]. Previous studies have demonstrated that neutralization of PAI-1 enhanced acute lung injury and alveolar hemorrhage with *in S. p*-infected mice [18]. In the current study, we showed that PAI-1 could be induced by *S. p* infection in multiple cell lines (A549, THP-1 and HeLa) with different background. Accumulating evidences have shown that CYLD is a potent negative regulator of pro-inflammatory cytokines induction [13,20,29]. CYLD exerts the effects through deubiquiting and in-activating Certain key molecule in the pro-inflammatory signalings [13,20,29]. Our evidences here demonstrated that CYLD inhibited *S. p*-stimulated PAI-1 expression. Over-expression of wt-CYLD dramatically inhibited PAI-1 mRNA expression in A549 cells with *S. p* infection. Reversely, introducing the mutant CYLD facilitated *S. p*-mediated PAI-1 expression.

Our *in vitro* results are consistent with previous *in vivo* observations showing that *S. p* infection-induced PAI-1 expression was remarkably increased in CYLD knockout mice, the latter were protected from acute lung injury [18]. Results from the same study using the CYLD knockout cells have showed that CYLD negatively regulates PAI-1 expression via inhibition of MKK3-p38 MAPK signaling pathway [18]. However, the detailed mechanisms were not known, and whether these was a direct interaction between CYLD and MKK3-p38 was not tested [18].

TRAF-6 is an ubiquitin ligase, and its activation also requires ubiquitination [26,30,31]. Studies have shown that TRAF-6 could serve as an upstream kinase of MKK3-p38 MAPK activation [32], it is also a target protein of CYLD [17]. Here, we provide evidences to support that TRAF-6 is required for *S. p*-induced PAI-1 expression. Our evidences include that siRNA-mediated knockdown of TRAF-6 remarkably inhibited *S. p*-induced PAI-1 expression. On the other hand, PAI-1 expression by *S. p* was further enhanced by TRAF-6 overexpression.

Significantly, we demonstrated that CYLD-mediated inhibition on PAI-1 might be due to its interaction with TRAF-6. Our co-IP and confocal microscopy assay results confirmed a direct association between the two. We showed that CYLD over-expression largely inhibited TRAF-6 ubiquitination, a step that is required for its activation. CYLD mutation, on the other hand, enhanced TRAF-6 ubiquitination in A549 cells. Based on these information, we

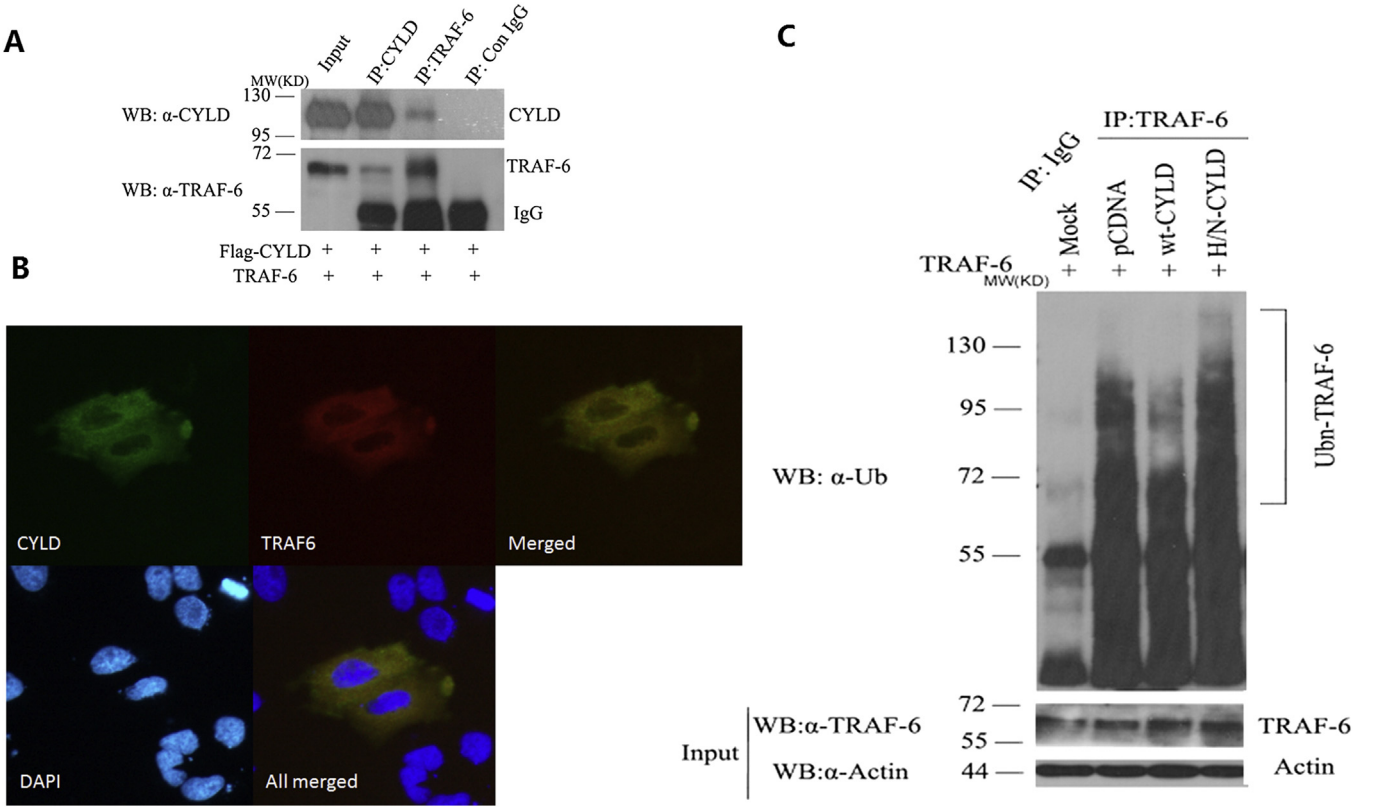

Fig. 3. CYLD works as a deubiquitinase to remove ubiquitin chains from TRAF-6 in A549 cells. Flag-CYLD plasmid and wt-TRAF-6 plasmid were co-transfected into A549 cells, CYLD-TRAF-6 co-localization was tested by Co-IP assay (A) and confocal microscopy assay (B). (C) A549 cells, transfected with empty vector, wt-TRAF-6 or plus wt- or H/N- CYLD, were treated with *S. p* lysates for 1 h, followed by Co-IP assaying of TRAF-6 ubiquitination, expression of TRAF-6 and α-actin in whole cell lysates was also tested as "Input" (C). Similar results were obtained from three independent experiments.

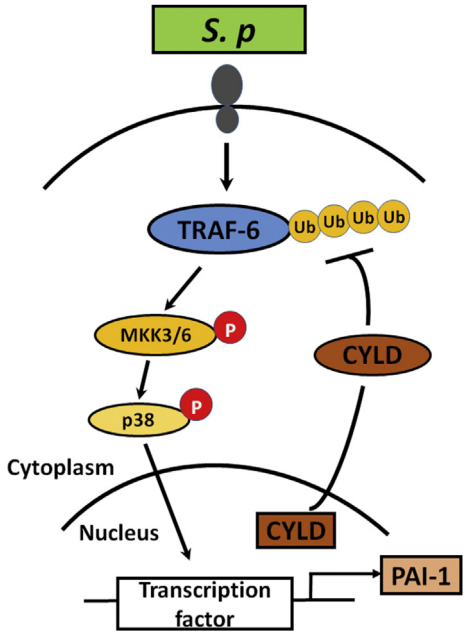

Fig. 4. Schematic representation of the negative regulation of PAI-1 expression by CYLD. TRAF-6 mediates PAI-1 expression by *S. p*. CYLD forms a complex with TRAF-6, and acts as a deubiquitinase to remove ubiquitin chains from TRAF-6, thus inhibiting TRAF-6 activation and subsequent PAI-1 expression.

propose that CYLD associates, deubiquinites and in-activates TRAF-6, thus inhibiting subsequent PAI-1 expression (see Fig. 4).

Thus, the results of this study suggest that TRAF-6 mediates *S. p*-induced PAI-1 expression, and CYLD inhibits such an effect probably through deubiquitinating TRAF-6. The current study provided molecular insights of CYLD-mediated activities in *S. p*-induced PAI-1 expression and possible acute lung injury [18].

Competing interests

The authors have no competing interests.

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Transparency document

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Appendix A. Supplementary data

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