Dong et al. *Zool. Res.* 2026, 47(5): 1471−1484
https://doi.org/10.24272/j.issn.2095-8137.2025.520
https://cstr.cn/32248.14.zr.2025.520

ZR | Zoological Research

Article Open Access

E3 ubiquitin ligase Smurf1 facilitates bacterial infection and regulates inflammatory responses via promoting MyD88 degradation in teleost

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ABSTRACT

In innate immunity, effective activation of the NF-κB signaling pathway is crucial for initiating inflammatory responses and eliminating invading bacteria, and its precise regulation relies heavily on the ubiquitin-proteasome system. Ubiquitination, a pivotal post-translational modification, is specifically catalyzed by E3 ubiquitin ligases that determine substrate selection. Ubiquitination of the adaptor protein MyD88 is critical for immune signaling; however, its regulatory mechanism in teleost fish, especially regarding the involvement of E3 ubiquitin ligases remains unclear. Among these E3 ubiquitin ligases, SMAD ubiquitylation regulatory factor 1 (Smurf1), while important in multiple signaling pathways, has not been reported to regulate antibacterial immunity in teleost fish. Thus, we systematically investigated Smurf1's role and molecular mechanism in this immune process, using *Miichthys miiuy* as a model. Reverse transcription-quantitative real-time polymerase chain reaction (RT-qPCR) analysis in *M. miiuy* kidney cells (MKC) revealed that Smurf1 overexpression suppressed pro-inflammatory cytokine expression and enhanced *Vibrio anguillarum* proliferation and adhesion, while Smurf1 knockdown exerted the opposite effects. Mechanistically, Smurf1 directly interacts with MyD88, promoting MyD88 proteasomal degradation through K48-linked polyubiquitination, which is dependent on Smurf1's E3 ligase activity (C704A) and HECT domain, as the C704A mutation or HECT domain deletion abolished this function. Luciferase reporter assays confirmed that Smurf1-mediated MyD88 degradation inhibits NF-κB signaling, thereby negatively regulating innate immunity and maintaining inflammatory homeostasis. Our findings reveal a mechanism by which Smurf1 balances immunity and homeostasis via MyD88 ubiquitination, providing valuable insights into vertebrate immune regulation.

Keywords: Smurf1; MyD88; NF-κB; *Vibrio anguillarum*; Ubiquitination

INTRODUCTION

The immune system is fundamentally divided into innate and adaptive immunity, with innate immunity serving as the primary defense against infections caused by pathogens in most organisms (Dinarello, 2018). Upon pathogen invasion, the innate immune system detects pathogen-associated molecular patterns (PAMPs) through pattern-recognition receptors (PRRs), including Toll-like receptors (TLRs), nucleotide-binding oligomerization domain (NOD)-like receptors (NLRs), and retinoic acid-inducible gene I (RIG-I)-like receptors (RLRs) (Akira et al., 2006; O'Neill & Bowie, 2007; Takeuchi & Akira, 2010). These receptors initiate innate immune responses and inflammatory cascades to combat invading pathogens. Among PRRs, TLRs are ancient and evolutionarily conserved components of the immune system, have been extensively studied for their critical role in coordinating both innate and adaptive immune responses (Janeway & Medzhitov, 2002).

The TLR signaling pathway represents a core signal transduction cascade in innate immunity, wherein TLRs serve as primary sensors for conserved pathogenic ligands. Upon ligand binding, TLRs activate either MyD88-dependent or MyD88-independent downstream pathways. Notably, all mammalian TLRs, with the exception of TLR3 rely on MyD88 for effective signal transduction (Takeda et al., 2003). In the MyD88-dependent pathway, ligand engagement triggers the sequential recruitment of MyD88, IL-1 receptor-associated kinases (IRAKs), and tumor necrosis factor receptor-

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Received: 22 October 2025; Accepted: 20 January 2026; Online: 23 January 2026

Foundation items: This work was supported by the Natural Science Foundation of Shanghai (25ZR1401163)

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associated factor 6 (TRAF6), ultimately leading to TAK1/IKK activation, NF-κB nuclear translocation, and production of inflammatory cytokines (e.g., TNF-α, IL-6), thus effectively mounting the host's innate immune response (Cao et al., 1996; Emmerich et al., 2013; West et al., 2006). Given that MyD88 plays a pivotal role in most TLR-mediated inflammatory responses, its activity is under tight control; dysregulation of MyD88 activity is a significant contributor to immune dysfunction and inflammatory diseases (Deguine & Barton, 2014; Kondo et al., 2012). Research has demonstrated that the stability of MyD88 is stringently regulated through diverse post-translational modifications (PTMs), including phosphorylation, ubiquitination (K48-/K63-linked), as well as autophagy-dependent protein turnover. In mammals, several E3 ubiquitin ligases, which include SPOP, Cbl-b, Smurf1, and Nrdp1, have been shown to modulate TLR signaling by regulating MyD88 protein abundance (Guillamot et al., 2019; Han et al., 2010; Lee et al., 2011; Li et al., 2020; Meng et al., 2021). Conversely, the deubiquitinating enzymes CYLD, OTUD4, and USP7 inhibit MyD88-mediated NF-κB signaling through their deubiquitination activities (Lee et al., 2016; Zhang et al., 2022; Zhao et al., 2018b). Similar to its function in mammals, MyD88 plays essential roles in innate immune responses in teleosts, and its protein stability is strictly regulated via ubiquitination.

Intracellular signaling initiated by innate immune receptors is a highly dynamic process that is regulated by various mechanisms, which collectively govern protein–protein interactions, subcellular localization, and protein abundance (Laney & Hochstrasser, 1999; Zinngrebe et al., 2014). Ubiquitination stands out as a pivotal mechanism within this process, playing a critical role in innate immune functions (Woelk et al., 2007). By precisely modulating the stability, activity, and localization of key components of PRR signaling pathways, this post-translational modification fine-tunes inflammatory responses and prevents tissue damage caused by excessive activation (Bhoj & Chen, 2009). The specificity and efficacy of ubiquitination fundamentally depend on the substrate recognition capacity of E3 ubiquitin ligases. SMAD ubiquitination regulatory factor 1 (Smurf1), classified as a HECT-type E3 ubiquitin ligase, has attracted increasing research interest due to its complex multi-domain structure and extensive regulatory functions across multiple signaling pathways. Structurally, Smurf1 consists of an N-terminal C2 domain that facilitates subcellular localization and membrane targeting, two WW domains responsible for recognizing substrate PY motifs, and a C-terminal HECT domain that confers catalytic activity (Weber et al., 2019). Initially identified for its role in targeting SMAD1/5 for proteasomal degradation in the bone morphogenetic protein (BMP) signaling pathway, Smurf1 is now recognized as a key regulator of innate immunity, including pathogen recognition and inflammatory signaling (Ingham et al., 2004). Smurf1's documented interactions with components such as MyD88, TRAF6, MEKK2, USP5, STAT1, and MAVS highlight its multifunctional role in immune regulation (Lee et al., 2011; Li et al., 2010; Ma et al., 2019; Peng et al., 2022; Qian et al., 2016; Wang et al., 2012, 2013; Yuan et al., 2012). While existing studies have clearly reported the regulatory role of Smurf1 in MyD88, EFHD2 has been shown to suppress TRAF6-mediated antiviral signaling by stabilizing Smurf1 in teleost fish (Wang et al., 2024; Zhang et al., 2022), which suggests that Smurf1 may broadly modulate the host immune response against various pathogens through a similar ubiquitination-dependent signaling pathway. However, whether Smurf1 mediates antibacterial immune responses in teleost fish and what its underlying molecular mechanism is remain entirely unknown.

This study identifies Smurf1 as a pivotal negative regulator of antibacterial immunity and inflammatory responses in teleost fish. We hypothesized that Smurf1 acts as an E3 ubiquitin ligase to promote K48-linked polyubiquitination and proteasomal degradation of MyD88, thereby inhibiting NF-κB activation. This hypothesis was confirmed in the miiuy croaker model, a finding that provides insights into how ubiquitination maintains teleost immune homeostasis.

MATERIALS AND METHODS

Animals and challenge

Miiuy croaker (*M. miiuy*, ~50g) were injected with *Vibrio. anguillarum* (centrifuged and resuspended to a concentration of approximately 3.0×10^7 colony forming units (CFU)/mL) or lipopolysaccharide (LPS, 10 μg/mL), as previously described (Zheng et al., 2023). Subsequently, the experimental fish were sacrificed at different time points, and kidney tissues were collected for RNA extraction. All animal experimental procedures were conducted in accordance with the Guidelines for the Care and Use of Laboratory Animals of the National Institutes of Health, and the experimental protocols were approved by the Research Ethics Committee of Shanghai Ocean University (No. SHOU-DW-2018-047).

Cell culture and transient transfection

For the present study, two fish cell lines, MKC and *Epithelioma papulosum cyprini* cells (EPC), were routinely cultured and maintained in our laboratory. MKC were cultured in L-15 medium (HyClone, USA) supplemented with 10% fetal bovine serum (FBS, Gibco), 100 U/mL penicillin (Gibco), and 100 μg/mL streptomycin at 26°C (Cui et al., 2022). EPC were grown at 28°C in a humidified incubator with 5% CO_2 in Medium 199 (Thermo Fisher Scientific, USA) supplemented with 10% FBS, 100 U/mL penicillin, and 100 mg/mL streptomycin. HEK293 cells were maintained at 37°C with 5% CO_2 in Dulbecco's Modified Eagle Medium (DMEM, HyClone) supplemented with 10% FBS, 100 U/mL penicillin, and 100 mg/mL streptomycin. For gene functional analysis, cells were transfected with the appropriate plasmids using Lipofectamine 3000 (Thermo Fisher Scientific, USA), according to the manufacturer's protocol. In the stimulation experiments, MKC cells were stimulated with *V. anguillarum* or LPS for different durations, and subsequently subjected to RT-qPCR and immunoblotting analysis. To investigate protein turnover, the proteasome inhibitor MG132 (20 mmol/L, Cat#S1748, Beyotime, China), the autophagy inhibitors 3-methyladenine (3-MA, 2 mmol/L, Cat#189490, Beyotime, China) and NH_4Cl (20 mmol/L, Cat#ST2030, Beyotime, China), or cycloheximide (CHX, 100 μg/ml, Cat#Y237498, Beyotime, China) were added to the culture medium 24 h after transfection.

Plasmid construction

The coding sequences (CDSs) of Smurf1, MyD88, IRAK1, IRAK4, and TRAF6 were amplified from *M. miiuy* cDNA by standard PCR and subsequently individually cloned into the pcDNA3.1 expression vector tagged with Flag, Myc, and HA tags. The Smurf1 gene domains were predicted using the Simple Modular Architecture Research Tool (SMART; http://smart.embl-heidelberg.de/). Site-directed mutagenesis

was performed to generate various Smurf1 mutants (Smurf1-ΔC2, Smurf1-ΔWW, Smurf1-ΔHECT, and Smurf1-C704A). These mutant fragments were amplified by PCR using specific primers and the Smurf1-Flag recombinant plasmid as the template. The expression plasmids pRK5-HA-ubiquitin-WT (ubiquitin-HA), ubiquitin-K48-HA, and ubiquitin-K63-HA were obtained from Addgene. All plasmids were extracted using the Endotoxin-Free Plasmid DNA Miniprep Kit (TIANGEN Biotech, China, Cat#DP123) and confirmed by Sanger sequencing. The primers used are listed in Supplementary Table S1.

RNA isolation and qPCR analysis

Total RNA was isolated from samples using TRIzol reagent (Takara Bio, Japan) according to the manufacturer's instructions. The FastQuant RT Kit (TIANGEN Biotech, China) was used to synthesize cDNA, which incorporates a DNase treatment step to eliminate potential genomic DNA contamination. RT-qPCR was performed on a QuantStudio 3 Real-Time PCR system (Applied Biosystems, USA) using the SYBR Premix Ex Taq Kit (Vazyme, China). The PCR cycling conditions were as follows: 30 s at 95°C, followed by 40 cycles of 10 s at 95°C, and 30 s at 60°C. β-actin was used as an internal reference. The primers used for RT-qPCR are listed in Supplementary Table S1. Three independent experiments were performed for the statistical analysis.

RNA interference (RNAi)

For transient silencing experiments, MKC were seeded into 24-well plates and cultured overnight. The cells were then transfected with siRNA or negative control siRNA (si-NC) using Lipofectamine RNAiMAX transfection reagent (Thermo Fisher Scientific, USA), following the manufacturer's protocol. The siRNA sequences targeting *M. miiuy* Smurf1 and the negative control were as follows: si-Smurf1-1:5′-CAG CGUUUAGAUCUAUGUTT-3′(sense) and 5′-ACAUAGAU CUAAACGCUGTT-3′(antisense), si-Smurf1-2:5′-CUUCGAC CAUAAAGAACUATT-3′(sense) and 5′-UAGUUCUUUAU GGUCGAAGTT-3′(antisense), si-NC (Negative Control): 5′-UU CUCCGAACGUGUCACGUTT-3′(sense) and 5′-ACGUGA CACGUUCGGAGAATT-3′ (antisense).

Luciferase reporter assays

EPC cells were co-transfected with expression plasmids and NF-κB, IL-8, or IL-1β reporter plasmids, together with the Renilla luciferase plasmid pRL-TK (Promega, USA) as an internal control at a 1:10 ratio (Chang et al., 2022). Luciferase activity was measured using the Dual-Luciferase Reporter Assay System (Promega, USA) according to the manufacturer's instructions. The control group was transfected with the same amounts of the corresponding empty vectors as the experimental group. Three independent experiments were performed, each in triplicate.

Western blotting

The cells were washed twice with ice-cold PBS and lysed. The lysates were centrifuged at 12 000 ×*g* for 15 min at 4°C. The supernatants were collected, and protein concentrations were determined using the bicinchoninic acid assay method (Pierce, USA) and balanced with cell lysis buffer. The samples were then subjected to SDS-PAGE and transferred to PVDF membranes (Millipore, USA) using semi-dry blotting (Bio-Rad Trans-Blot Turbo System, USA). Subsequently, the membranes were blocked with 5% non-fat dry milk in TBST (20 mmol/L Tris, 150 mmol/L NaCl, 0.1% Tween 20, pH 7.5) for 1.5 h at room temperature. After a brief wash with TBST, the membranes were incubated overnight at 4°C with the following primary antibodies: anti-Flag (Cat#AF2852, Beyotime, China, 1:1 000), anti-Myc (Cat#AF2867, Beyotime, China, 1:1 000); anti-HA (Cat#AF2858, Beyotime, China, 1:1 000); anti-Tubulin (Cat#AF2827, Beyotime, China, 1:1 000) and anti-MyD88 (Cat#PB9148, Boster, China, 1:500). The following day, the membranes were washed thrice with TBST and incubated with horseradish peroxidase (HRP)-conjugated secondary antibodies (Beyotime, China) for 1.5 h at room temperature. After extensive washing, immunoreactive bands were visualized using WesternBright ECL substrate (Advansta, USA) and imaged using a cooled CCD camera system (Tanon, China).

Co-IP assay

HEK293 or EPC cells were seeded in 10-cm^2 dishes and grown overnight. Cells were co-transfected with a total of 6 μg plasmids. At 36 h post-transfection, cells were washed three times with ice-cold PBS and lysed in IP lysis buffer (Beyotime, China) supplemented with protease inhibitor cocktail and PMSF for 20 min at 4°C with gentle rocking. Cellular debris was removed by centrifugation at 12 000 ×*g* for 10 min at 4°C. The supernatant was incubated with 1 μg of the indicated primary antibody (Beyotime, China) for 2.5 h at 4°C, followed by the addition of Protein A/G PLUS-Agarose beads (Santa Cruz Biotechnology, USA) and incubation for an additional 10 h at 4°C. The beads were then collected by centrifugation at 2 500 ×*g* for 5 min at 4°C and washed three times with NP-40 lysis buffer (5 min per wash) with gentle inversion on ice. Bound proteins were eluted in 2× SDS loading buffer by boiling for 10 min and analyzed by immunoblotting using the appropriate antibodies.

Ubiquitination assay

Ubiquitination assays were performed as previously described (Chen et al., 2023). Cells cultured in 10 cm^2 dishes were transfected with the specified plasmids for 36 h. The cells were then washed with ice-cold PBS, harvested, and divided into two aliquots. One aliquot was lysed in NP-40 lysis buffer and analyzed by Western blotting to determine the input protein levels. The other aliquot was subjected to immunoprecipitation under denaturing conditions to capture ubiquitinated proteins, which were then analyzed by immunoblotting using the relevant antibodies.

Bacterial adhesion assay

Cells were infected with *V. anguillarum* (1×10^6 cells/mL) for 2 h, and the medium was then aspirated. Cells were washed three times with PBS and digested with trypsin for 3–5 min. The trypsin was discarded, and cells were scraped into PBS. Cells were centrifuged at 12 000 ×*g* for 5 min. After centrifugation, the supernatant was discarded, and cell lysis buffer was added and mixed uniformly with the cell pellet. Following a 30 min incubation at room temperature, the lysate was serially diluted 1 000-fold in PBS and spread onto TSB agar plates. The plates were incubated at 28°C for 12 h, and bacterial colonies were counted to determine the number of colony-forming units (CFU).

Statistical analysis

Statistical analyses were performed using Student's *t*-test. The $2^{-\Delta\Delta CT}$ method was utilized to acquire relative gene expression levels, and one-way analysis of variance (ANOVA) combined with Duncan's multiple comparison tests was used to analyze intergroup differences (Livak & Schmittgen, 2001). Data are

presented as mean±standard error (SE) from at least three independent triplicated experiments. *: $P<0.05$ and **: $P<0.01$ were considered statistically significant.

RESULTS

Smurf1 inhibits innate immune response upon *V. anguillarum* infection

Innate immunity serves as the first line of defense against bacterial invasion. Previous studies have demonstrated that Smurf1 plays a critical role in antiviral innate immunity in teleosts (Wang et al., 2024). However, its specific functions and molecular mechanisms in antibacterial immune responses remain unclear in teleosts. Given the substantial economic losses caused by *Vibrio* infections in high-density aquaculture, we investigated the role of Smurf1 in antibacterial immunity using the teleost fish *M. miiuy*. To characterize its expression pattern during pathogen challenge, we quantified Smurf1 transcripts in kidney tissues and MKC cells following *V. anguillarum* infection via RT-qPCR. The results showed that Smurf1 expression was significantly upregulated in both kidney tissues and MKC cells after infection (Figure 1A). Since LPS, an endotoxin derived from Gram-negative bacteria, is known to maintain bacterial structural integrity and provoke potent immune responses, we employed LPS as PAMP to simulate bacterial infection. Consistent with the bacterial challenge, LPS stimulation significantly increased Smurf1 transcriptional levels in both kidney tissues and MKC cells (Figure 1B). Thus, it is hypothesized that Smurf1 may be involved in innate immunity. To explore the biological function of Smurf1, we designed small interfering RNAs (siRNAs) targeting Smurf1. We verified the Smurf1 knockdown efficiency at both the mRNA level and protein level. The experimental results showed that the designed small siRNAs (si-Smurf1-1 and si-Smurf1-2) significantly downregulated Smurf1 mRNA and protein expression levels of Smurf1 (Figure 1C). Using the most efficient siRNA, we found that Smurf1 knockdown significantly inhibited *V. anguillarum* infection in MKC cells (Figure 1D). To further explore whether Smurf1 is involved in the host infection process, bacterial adhesion assays revealed that Smurf1 knockdown reduced the number of *V. anguillarum* CFUs adhering to MKC cells (Figure 1E, F), indicating its involvement in the early stages of host infection. Given the crucial role of inflammatory cytokines in host defense, we next examined whether Smurf1 regulates innate immune responses by modulating their expression. Upon stimulation with LPS or *V. anguillarum*, Smurf1 overexpression significantly suppressed the mRNA levels of TNF-α, IL-1β, IL-6, and IL-8, whereas its knockdown enhanced the induction of these pro-inflammatory cytokines (Figure 1G–I). In conclusion, our results demonstrate that the E3 ubiquitin ligase Smurf1 acts as a negative regulator of host antibacterial immunity and inflammatory responses.

Smurf1 suppresses NF-κB activation induced by key components of the MyD88-dependent pathway

NF-κB is a pivotal transcription factor in innate immune responses, and the mechanisms governing its regulation have attracted considerable research interest. In the study, we explored the regulatory function of Smurf1 within the NF-κB signaling pathway through concentration- and time-gradient experiments. EPC cells were co-transfected with Smurf1 overexpression plasmids along with NF-κB, IL-8, and IL-1β reporter gene plasmids, followed by LPS stimulation. An untreated control group was included, in which cells were co-transfected with pRL-TK, pcDNA3.1, and reporter plasmids. As shown in Figure 2A–C, Smurf1 overexpression significantly suppressed NF-κB signaling and the promoter activity of its downstream inflammatory cytokines, providing key experimental evidence for its role in regulating inflammatory responses. In mammals, TLR4 recognizes LPS and initiates the MyD88-dependent pathway, a core mechanism for antibacterial defense (Valanne et al., 2011). Upon pathogen recognition, MyD88 is recruited to all TLRs except TLR3, and binds to IRAKs and TRAF6 to transmit signals. While TLR4 may be absent or unable to effectively recognize LPS in lower vertebrates, the high diversity of the TLR family in these species may potentially compensate for this functional deficit (Liu et al., 2010; Sepulcre et al., 2009). Notably, the core TLR signaling modules are highly conserved from invertebrates to mammals (Akira et al., 2003; Purcell et al., 2006). This conservation underscores the evolutionary significance of TLR-mediated immunity and implies that MyD88 likely remains a central signaling hub in teleosts, thereby representing a potential target for Smurf1 in the regulation of antibacterial immunity (Chen et al., 2022). To identify targets of Smurf1, we evaluated its effect on key components of the MyD88-dependent signaling cascade. As shown in Figure 2D, Smurf1 overexpression significantly inhibited NF-κB promoter activity induced by key MyD88-interacting molecules (IRAK1, IRAK4, TRAF6) and the expression of its downstream inflammatory cytokines. To identify the primary target underlying this broad inhibition, we hypothesized that targeting the upstream adaptor MyD88 would most directly explain the concomitant suppression of downstream events. As TRAF6 was already known as a target of Smurf1 in antiviral immunity, we focused on MyD88 to probe antibacterial mechanism (Wang et al., 2024). Thus, MyD88 was prioritized for in-depth analysis.

Smurf1 interacts with MyD88 and facilitates the degradation of MyD88

MyD88 is a central adaptor in most TLR signaling pathways, and its protein levels must be tightly regulated to maintain immune homeostasis. Initially, using a dual-luciferase reporter assay, we found that Smurf1 overexpression suppressed NF-κB activation induced by key components of the MyD88-dependent signaling cascade, including IRAK1, IRAK4, and TRAF6 (Figure 2D). Since MyD88 serves as an upstream hub whose stability dictates downstream signaling, we hypothesized that Smurf1 negatively regulates NF-κB pathway by potentially targeting MyD88 for degradation. To test this hypothesis, we conducted a series of functional validation experiments. Importantly, Co-IP assays performed in both HEK293 and EPC cells confirmed a direct molecular interaction between MyD88 and Smurf1, providing direct evidence for their functional association (Figure 3A, B). Furthermore, dose-dependent and time-course experiments showed that increasing Smurf1 expression reduced MyD88 protein levels, suggesting that Smurf1 promotes MyD88 degradation (Figure 3C, D). Consistently, Smurf1 knockdown using siRNA significantly delayed the degradation of endogenous MyD88 protein (Figure 3E, left panel) and enhanced LPS-induced MyD88 transcriptional levels (Figure 3E, right panel). Notably, this delay in MyD88 degradation was more pronounced following LPS treatment (Figure 3F). Together, these data indicate that Smurf1

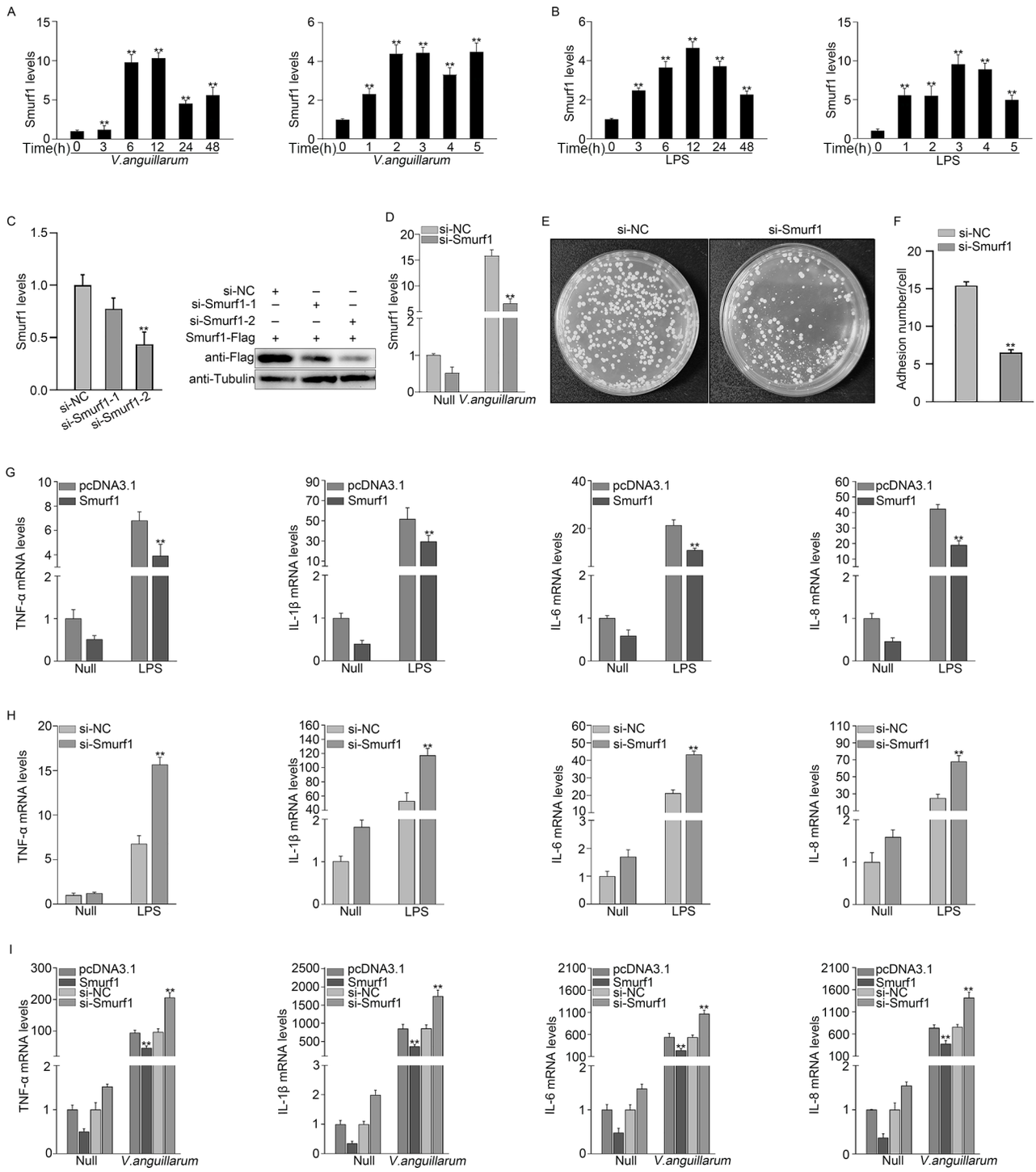


Figure 1 Smurf1 inhibits innate immune response upon *V anguillarum* infection

A, B: At different time points, RT-qPCR was used to determine the mRNA expression levels of the Smurf1 gene in the kidney tissues of *M. miiuy* and in MKC cells after infection with *V. anguillarum* (A) or LPS (B). C: MKCs were seeded in 24-well plates and cultured overnight, then transfected with 100 nmol/L si-NC, si-Smurf1-1, or si-Smurf1-2. RT-qPCR was used to determine Smurf1 mRNA level expression (left); Western blot was used to determine Smurf1 protein level expression (right). D:RT-qPCR analysis of Smurf1 mRNA levels in si-NC or si-Smurf1 transfected MKCs with or without *V. anguillarum* infection. E, F: MKCs were first transfected with si-NC or si-Smurf1, then infected with *V. anguillarum*. After infection, lysates of the infected MKCs were spread on LB agar plates and incubated for 12 h (E). Colony-forming units (CFU) were counted to evaluate the bacterial load in MKCs (F). G–I: RT-qPCR analysis of TNF-α, IL-1β, IL-6, and IL-8 mRNA levels in MKCs overexpressing 500 ng of pcDNA3.1 or Smurf1. At 24 h post-transfection, cells were stimulated with LPS for 12 h (G), transfected with 50 nM si-NC or si-Smurf1 under LPS stimulation for 12 h (H), or subjected to different treatments (pcDNA3.1, Smurf1, si-NC, si-Smurf1). At 24 h post-transfection, cells were stimulated with *V. anguillarum* (I). All data are presented as the mean±SE from at least three independent triplicated experiments. **: $P<0.01$.

specifically exerts a negative regulatory effect on MyD88. To further investigate whether Smurf1 regulates the MyD88-mediated NF-κB pathway, we co-transfected EPC cells with reporter plasmids, MyD88-Myc, and increasing amounts of

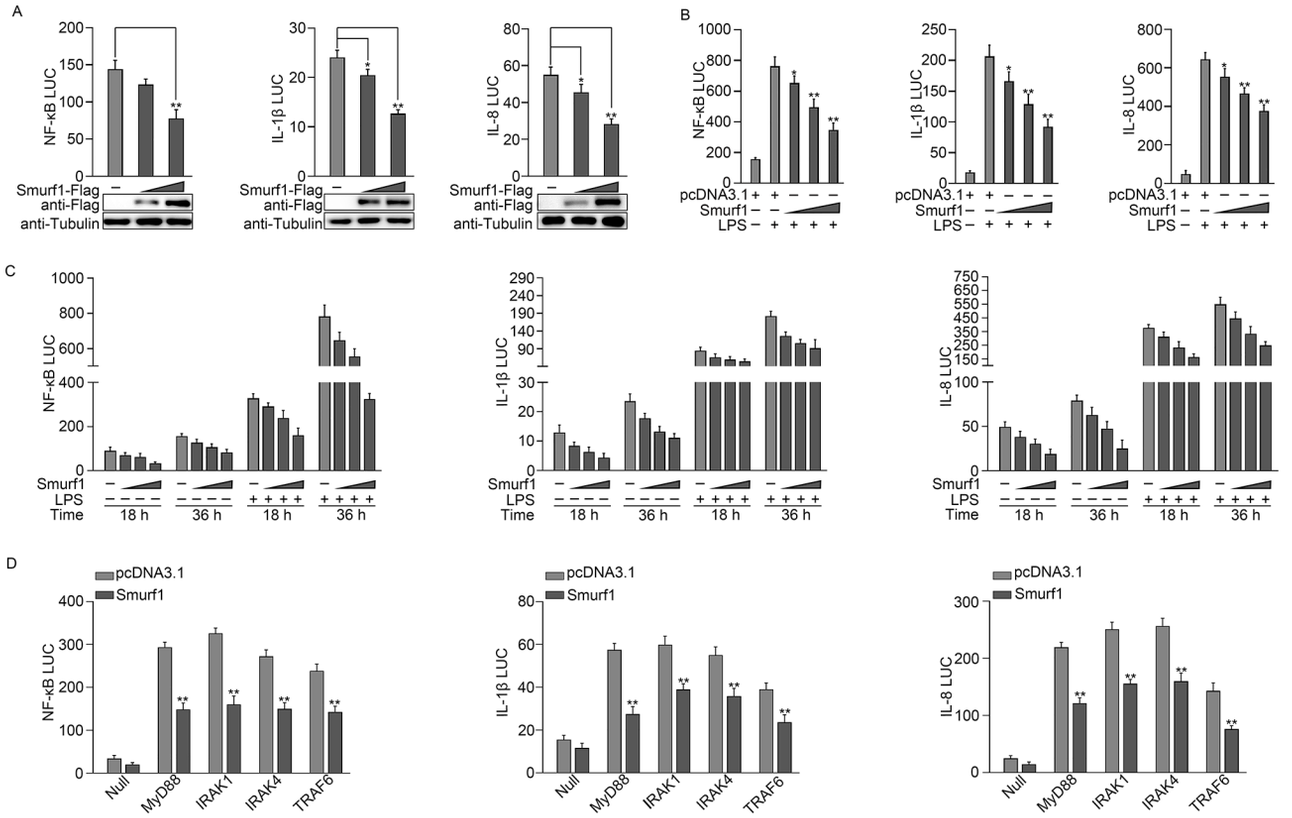


Figure 2 Smurf1 regulates the NF-κB signaling pathway and the expression of key signaling molecules

A, B: EPC cells were co-transfected with different concentrations of Smurf1 overexpression plasmid (0.1, 0.2 μg) (A); (0.1, 0.2, 0.3 μg) (B) or an equivalent amount of pcDNA3.1, along with 0.015 μg of pRL-TK plasmid and 0.15 μg of luciferase reporter plasmids driven by the NF-κB, IL-1β, or IL-8. After 48 h of transfection, cells were further stimulated with LPS for 12 h (B). Dual-luciferase reporter assay was then performed to measure the relative luciferase activity, reflecting the transcriptional activity of NF-κB, IL-1β, and IL-8. C: Time-course experiments were performed in EPC cells with or without LPS treatment, in which the cells were co-transfected with Smurf1 plasmids at different concentrations (0.1, 0.2, or 0.3 μg) or an equivalent amount of pcDNA3.1 plasmid, 0.015 μg of pRL-TK plasmid, and 0.15 μg of NF-κB, IL-1β, or IL-8 luciferase reporter plasmid. Luciferase activity was normalized to Renilla luciferase activity. D: EPC cells were seeded in 24-well plates and co-transfected with 0.1 μg of IRAK1, IRAK4, TRAF6, or MyD88 plasmid, together with Smurf1 plasmid or pcDNA3.1 plasmid, 0.15 μg of NF-κB, IL-1β, or IL-8 luciferase reporter plasmid, and 0.015 μg of pRL-TK plasmid. At 48 h post-transfection, luciferase activity was measured. All data represent results from at least three independent triplicated experiments. *: P<0.05; **: P<0.01.

Smurf1-Flag. The results showed that Smurf1 dose-dependently suppressed MyD88-induced NF-κB activation, an effect that was further enhanced by LPS stimulation (Figure 3G, H). Time-course experiments also confirmed the sustained suppression of MyD88 mediated NF-κB signaling by Smurf1 (Figure 3I, J).

Smurf1 promotes proteasomal degradation of MyD88

To elucidate the mechanism by which Smurf1 promotes MyD88 degradation, we first investigated whether Smurf1 regulates MyD88 protein stability. We found that Smurf1 overexpression significantly reduced MyD88 stability, whereas Smurf1 knockdown enhanced it (Figure 4A, B). Our analysis demonstrated that *V. anguillarum* infection induced MyD88 mRNA expression at various time points, and this effect was further enhanced by Smurf1 knockdown (Figure 4C). To assess whether Smurf1 regulates MyD88 stability at the post-translational level, we examined MyD88 degradation. We found that Smurf1 knockdown delayed endogenous MyD88 degradation in a concentration- and time-dependent manner following *V. anguillarum* infection (Figure 4D, E). To identify the degradation pathway, we used pharmacological inhibitors. This approach revealed that the proteasome inhibitor MG132 effectively blocked Smurf1-mediated MyD88 degradation, whereas the autophagy inhibitor 3-MA and lysosomal inhibitor NH_4Cl showed no effect (Figure 4F). A dose-response experiment further confirmed that MG132 inhibited Smurf1-mediated MyD88 degradation in a dose-dependent manner (Figure 4G). Collectively, these findings establish that Smurf1 accelerates MyD88 degradation by targeting it for proteasomal degradation, prompting us to next elucidate the underlying molecular mechanism.

Smurf1 promotes K48-linked polyubiquitination of MyD88 dependent on its E3 ligase activity

Ubiquitination plays a pivotal role in proteasomal degradation (Nandi et al., 2006). We investigated whether Smurf1, an E3 ligase, ubiquitinates MyD88. We found that Smurf1 overexpression promoted MyD88 ubiquitination (Figure 5A). To determine the specific type of ubiquitin linkage catalyzed by Smurf1, we used ubiquitin mutants containing K48O ubiquitin and K63O ubiquitin. (Figure 5B). Notably, in EPC cells, Smurf1 specifically promoted K48-linked ubiquitination of MyD88, as evidenced by enhanced ubiquitination in the presence of K48O ubiquitin. In contrast, neither the K63O nor the K48R mutant supported Smurf1-mediated ubiquitination of MyD88, underscoring the specificity of Smurf1 for the K48-linked ubiquitination of MyD88 (Figure 5C). To further confirm,

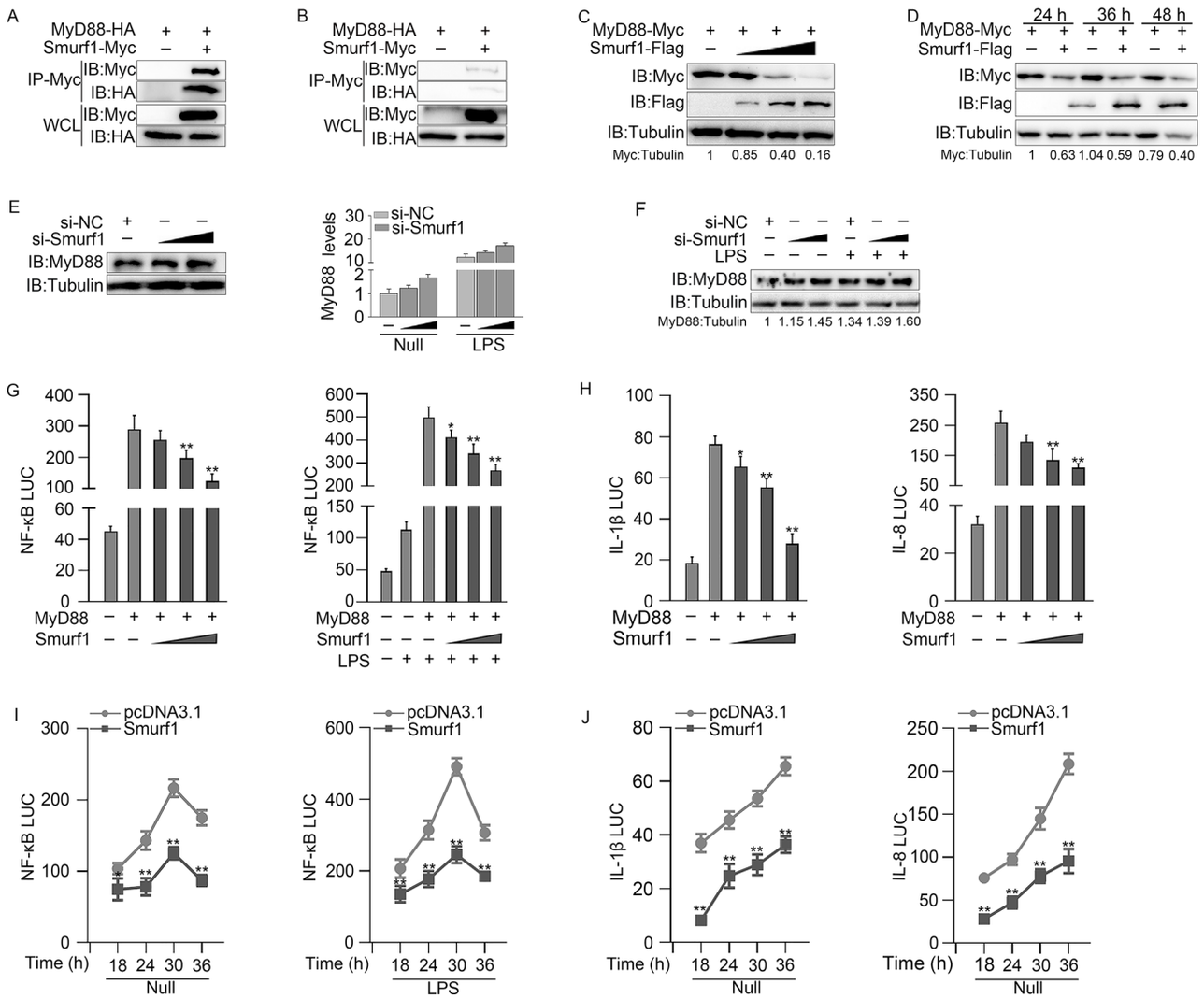


Figure 3 Smurf1 promotes the degradation of MyD88 and inhibits MyD88-mediated NF-κB signaling pathway

A, B: Co-IP and WB assays to detect the interaction between Smurf1 and MyD88. HEK293 cells (A) or EPC cells (B) were seeded in 6 cm^2 culture dishes and cultured overnight, then co-transfected with 2 μg of MyD88-HA plasmid and Smurf1-Myc plasmid or pcDNA3.1. At 48 h post-transfection, Co-IP was performed using anti-Myc antibody to pull down Smurf1-Myc, followed by WB analysis with anti-HA and anti-Myc. C: EPC cells seeded in 12-well plates were co-transfected with 0.5 μg of MyD88-Myc plasmid and Smurf1-Flag plasmid (0.1, 0.2, or 0.4 μg). The protein level of MyD88-Myc was determined by immunoblot assay. D: EPC cells in 12-well plates were co-transfected with 0.5 μg of MyD88-Myc plasmid and 0.4 μg of Smurf1-Flag plasmid. At 24, 36, and 48 h post-transfection, MyD88-Myc protein expression was detected by WB and normalized to Tubulin. E: MKC cells were transfected with 50 or 100 nmol/L si-Smurf1 or si-NC (left panel). WB assay was used to detect MyD88 protein level (right panel). After transfection, cells were stimulated with LPS for 12 h, and RT-qPCR was performed to measure relative MyD88 mRNA level. F: MKC cells were transfected with 50 or 100 nmol/L si-Smurf1 or si-NC. At 24 h post-transfection, cells were either stimulated with LPS for 12 h or left untreated. WB assay was used to determine the expression level of endogenous MyD88 protein. G, H: Cells were co-transfected with Smurf1-Flag plasmid (0.1, 0.2, or 0.3 μg), MyD88-Myc plasmid (0.1 μg), 0.015 μg of pRL-TK plasmid, and 0.15 μg of NF-κB, IL-1β, or IL-8 luciferase reporter plasmids. pcDNA3.1 was used to equalize total plasmid mass across groups. Relative luciferase activity was measured: at 48 h post-transfection (G, left panel; H) and 48 h post-transfection followed by stimulation with 10 μg/mL LPS for 12 h (G, right panel). I, J: Time-course experiments were performed following transfection with Smurf1. After transfection with Smurf1, luciferase activities of NF-κB, IL-1β, and IL-8 reporters were measured at different time points. All luciferase activities were normalized to Renilla luciferase activity. All data are presented as the mean±SE from at least three independent triplicated experiments. *: P<0.05; **: P<0.01.

we noted that this modification led to proteasomal degradation, as inhibition by MG132 resulted in the accumulation of ubiquitinated MyD88. Moreover, Smurf1 enhanced K48-linked ubiquitination of MyD88 in a concentration-dependent manner (Figure 5D). Consistent with human studies, the cysteine residue at position 704 (C704) within the C lobe of the HECT domain in *M. miiuy* Smurf1 is a conserved catalytic site, which is essential for forming a thioester intermediate with ubiquitin (Lin et al., 2023). Consequently, we generated the Smurf1-C704A mutant as an enzymatically inactive form to validate its role in regulating MyD88 degradation. To examine whether Smurf1-mediated degradation of MyD88 depends on its E3 ligase activity, we overexpressed wild-type Smurf1 and its catalytically inactive mutant Smurf1-C704A. Western blotting analysis revealed that wild-type Smurf1 promoted MyD88 degradation, whereas Smurf1-C704A restored MyD88 protein levels, indicating that Smurf1's E3 ligase activity is essential for MyD88 protein

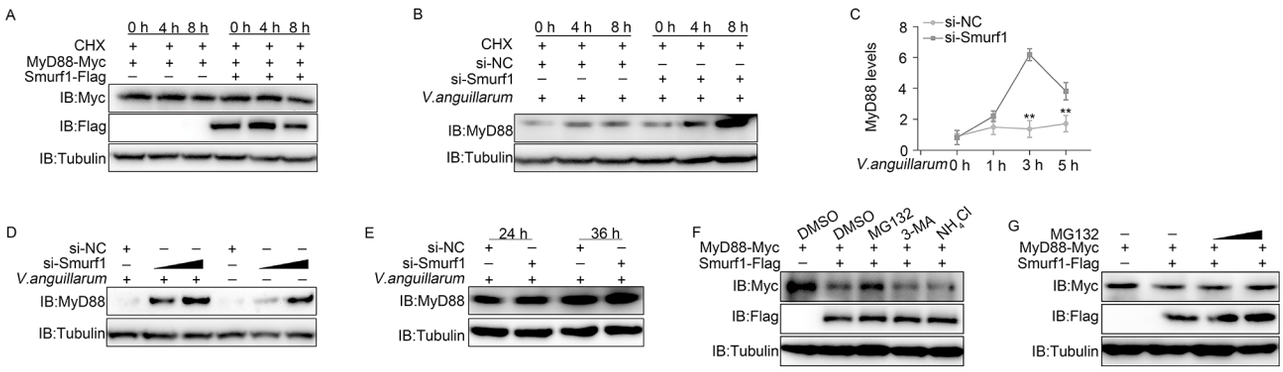


Figure 4 Smurf1 mediates MyD88 degradation via the ubiquitin-proteasome pathway

A: EPC cells were seeded in 12-well plates and cultured overnight, then co-transfected with 0.5 μg of MyD88-Myc plasmid and 0.5 μg of either pcDNA3.1 or Smurf1-Flag plasmid. At 24 h post-transfection, cells were treated with cycloheximide (CHX, 100 μg/mL) and harvested at distinct time points (0, 4, or 8 h) for subsequent analysis. Cell lysates were then analyzed by IB using antibodies against Flag, Myc, and Tubulin. B: MKCs were transfected with si-Smurf1 (Smurf1-specific siRNA); at 24 h post-transfection, cells were treated with 100 μg/mL CHX and subsequently infected with *V. anguillarum*. Cell lysates were collected at designated time points and analyzed by IB to detect MyD88 protein levels. C: MKCs were transfected with si-Smurf1 or si-NC for 24 h, followed by infection with *V. anguillarum* at different time points. Total RNA was extracted to determine the mRNA level of MyD88 using RT-qPCR. The relative mRNA level of MyD88 was normalized to that of β-actin and expressed as the fold induction relative to the control group. Data are presented as the mean±standard deviation (SD) from three independent experiments. D, E: Concentration- and time-dependent effects of Smurf1 knockdown on MyD88 protein levels under *V. anguillarum* infection. MKCs were transfected with different concentrations of si-Smurf1 (D); MKCs were transfected with si-Smurf1 and infected with *V. anguillarum* for different durations (E). At 24 h post-transfection (D) or designated infection time points (E), cells were further treated with *V. anguillarum* for 4 h (D) or harvested directly (E). IB analysis was performed using anti-MyD88 and anti-Tubulin antibodies, verifying whether Smurf1 regulates MyD88 in a concentration- or time-dependent manner. F: EPC cells in 24-well plates were co-transfected with 0.2 μg MyD88-Myc, 0.2 μg Smurf1-Flag, or pcDNA3.1. At 36 h post-transfection, cells were treated with 20 μmol/L MG132, 2 mmol/L 3-MA, 20 mmol/L NH_4Cl, or DMSO (control) for 8 h. Cell lysates were analyzed by IB with anti-Flag, anti-Myc, and anti-tubulin antibodies. G: EPC cells were co-transfected with 0.2 μg MyD88-Myc and 0.2 μg Smurf1-Flag (or pcDNA3.1). At 36 h post-transfection, cells were treated with 20 or 40 μmol/L MG132 for 8 h. Lysates were analyzed by IB with anti-Myc, anti-Flag, and anti-Tubulin antibodies. **: $P<0.01$.

degradation (Figure 5E). This rescuing effect of the C704A mutant on MyD88 abundance was further enhanced upon LPS stimulation, suggesting that Smurf1's regulatory influence on MyD88 stability is amplified under innate immune activation (Figure 5F). Interestingly, we also observed that wild-type Smurf1 suppressed MyD88 transcription upon immune stimulation, and this repression was reversed by the C704A mutant (Figure 5G), indicating an additional E3 ligase-dependent transcriptional regulatory layer. Crucially, in ubiquitination assays, wild-type Smurf1 enhanced K48-linked polyubiquitination of MyD88, while the C704A mutant abolished this activity (Figure 5H). Collectively, these findings demonstrate that Smurf1's catalytic activity is indispensable for MyD88 ubiquitination and degradation. Furthermore, Smurf1 exerts control over MyD88, operating at both transcriptional and post-translational levels in an E3 ligase activity-dependent manner. Subsequently, having validated that the catalytic activity of Smurf1 is essential for the regulation of MyD88, we further investigated the specific protein domain underlying this regulatory process.

The E3 ubiquitin ligase activity of Smurf1 is indispensable for the suppression of NF-κB signaling

To define the role of Smurf1's catalytic activity in the NF-κB-mediated inflammatory response, we conducted a series of functional assays. Initially, RT-qPCR analysis showed that upon stimulation with LPS or *V. anguillarum*, overexpression of wild-type Smurf1 (Smurf1-WT) significantly suppressed the transcription of inflammatory cytokines (TNF-α, IL-1β, IL-6, IL-8), whereas the catalytically inactive mutant Smurf1-C704A abrogated this inhibitory capacity (Figure 6A, B). Luciferase reporter assays further confirmed that Smurf1-WT, but not the Smurf1-C704A mutant, markedly inhibited LPS-induced activation of the NF-κB, IL-1β, and IL-8 promoters (Figure 6C). Time-course experiments demonstrated that this suppression by Smurf1-WT was sustained at both 18 h and 36 h post-stimulation, whereas the Smurf1-C704A mutant failed to exert any long-term inhibitory effect, underscoring the essential role of catalytic activity in pathway repression (Figure 6D). To determine whether the catalytic activity of Smurf1 is required for regulating bacterial adhesion, we overexpressed either Smurf1-WT or Smurf1-C704A in MKC cells. Bacterial adhesion assays were then performed to quantify the CFUs of *V. anguillarum* adherent to these cells. The results revealed that overexpression of Smurf1-WT significantly enhanced bacterial adhesion compared with the control group. In contrast, cells overexpressing Smurf1-C704A showed no increase in bacterial adhesion, with CFU levels similar to those of the control. These results indicate that the catalytic activity of Smurf1 is essential for promoting bacterial adhesion to host cells (Figure 6E). Subsequently, we assessed the functional role of the HECT domain, based on its identification as the location of C704. In summary, these findings collectively demonstrate that the catalytic activity of Smurf1 plays a critical negative regulatory role in the NF-κB pathway-mediated inflammatory response.

The HECT domain of Smurf1 is critical for the degradation of MyD88

To identify the key domains of Smurf1 responsible for regulating MyD88 stability, we first performed a domain structure analysis. Based on the human Smurf1 protein, we successfully constructed three domain-deletion mutants, including Smurf1-△C2 (in which the N-terminal C2 domain is

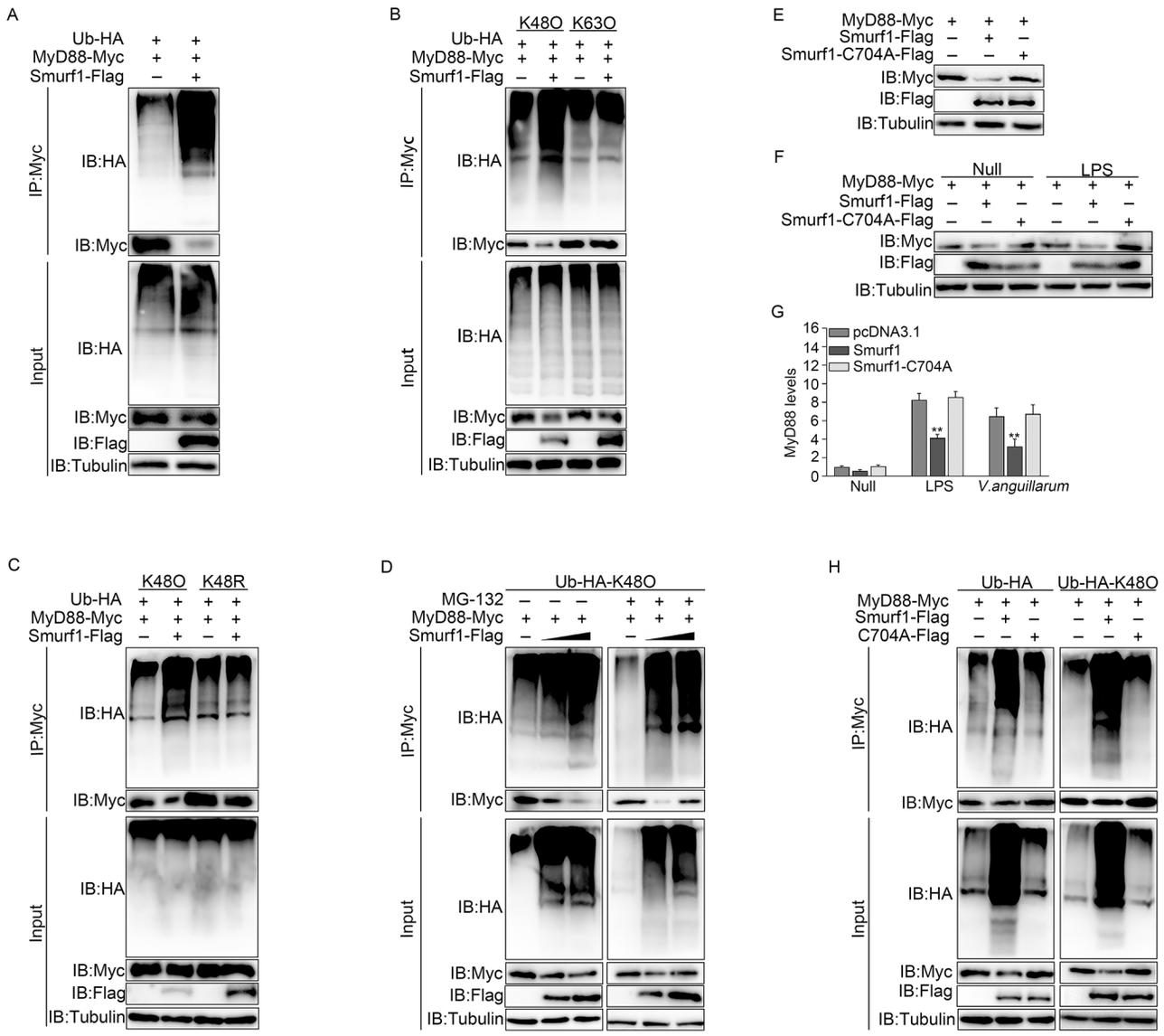


Figure 5 Smurf1 mediates K48-linked ubiquitination of MyD88 in an E3 ligase activity-dependent manner

A–D: EPC cells seeded in 6-cm^2 culture dishes underwent co-transfection with 2 μg of Smurf1-Flag plasmid (or pcDNA3.1 empty vector as a control), 2 μg of Ub-HA plasmid (A), along with Ub-HA-K48 or Ub-HA-K63 plasmid (B), or Ub-HA-K48O (retains K48-linked polyubiquitination) or Ub-HA-K48R (abrogates K48-linked polyubiquitination) plasmid (C), or Ub-HA-K48O and 20/40 μM MG132 for 6 h at 48 h post-transfection (D), and 1.5 μg of MyD88-Myc plasmid. 48 h following transfection, cell lysates were subjected to IB analysis using primary antibodies HA, Myc, Flag, and Tubulin. E, F: EPC cells were co-transfected with 0.5 μg of MyD88-Myc plasmid, along with 0.5 μg of Smurf1-Flag plasmid, 0.5 μg of Smurf1-C704A plasmid (E), or pcDNA3.1 plasmid. At 48 h post-transfection, cells were treated with LPS for 12 h (F), cell lysates were subjected to IB analysis using primary antibodies Myc, Flag, and Tubulin. G: MKC cells were transfected with control vector, Smurf1 plasmid, or Smurf1-C704A plasmid. After transfection, cells were treated with LPS or infected with *V. anguillarum*. RT-qPCR assays were performed to determine the mRNA expression levels of MyD88. Data are presented as mean±standard error (SE) from at least three independent triplicated experiments. H: EPC cells seeded in 6-cm^2 culture dishes were co-transfected with 2 μg of Smurf1-Flag plasmid, Smurf1-C704A-Flag plasmid, or pcDNA3.1 empty vector; 2 μg of Ub-HA plasmid or Ub-HA-K48O plasmid; and 1.5 μg of MyD88-Myc plasmid. At 48 h post-transfection, cell lysates were subjected to IB analysis using primary antibodies against HA, Myc, Flag, and Tubulin. **: P<0.01.

deleted), Smurf1-$^{\triangle}$WW (in which both WW domains are deleted), and Smurf1-$^{\triangle}$HECT (in which the HECT domain is deleted) (Figure 7A) (Dong et al., 2023). The construction of these mutants allowed us to determine the contribution of each domain to MyD88 stability. We co-transfected EPC cells with MyD88 and either wild-type Smurf1 or its three mutants. The results demonstrated that deletion of the HECT domain abrogated the ability of Smurf1 to regulate MyD88 (Figure 7B). In addition, Co-IP assay results demonstrated that MyD88 interacted with each Smurf1 domain-deletion mutant (Supplementary Figure S1A). Consistent with this, luciferase reporter assays revealed that the Smurf1-ΔHECT mutant lost the capacity to suppress NF-κB, IL-1β, and IL-8 reporter activity (Figure 7C). These assays were conducted using a dose-gradient design (Supplementary Figure S1B). Collectively, these data, together with the preceding findings, demonstrate that the E3 ubiquitin ligase Smurf1 promotes K48-linked ubiquitination of MyD88 and targets it for degradation via the ubiquitin-proteasome pathway (Figure 7D).

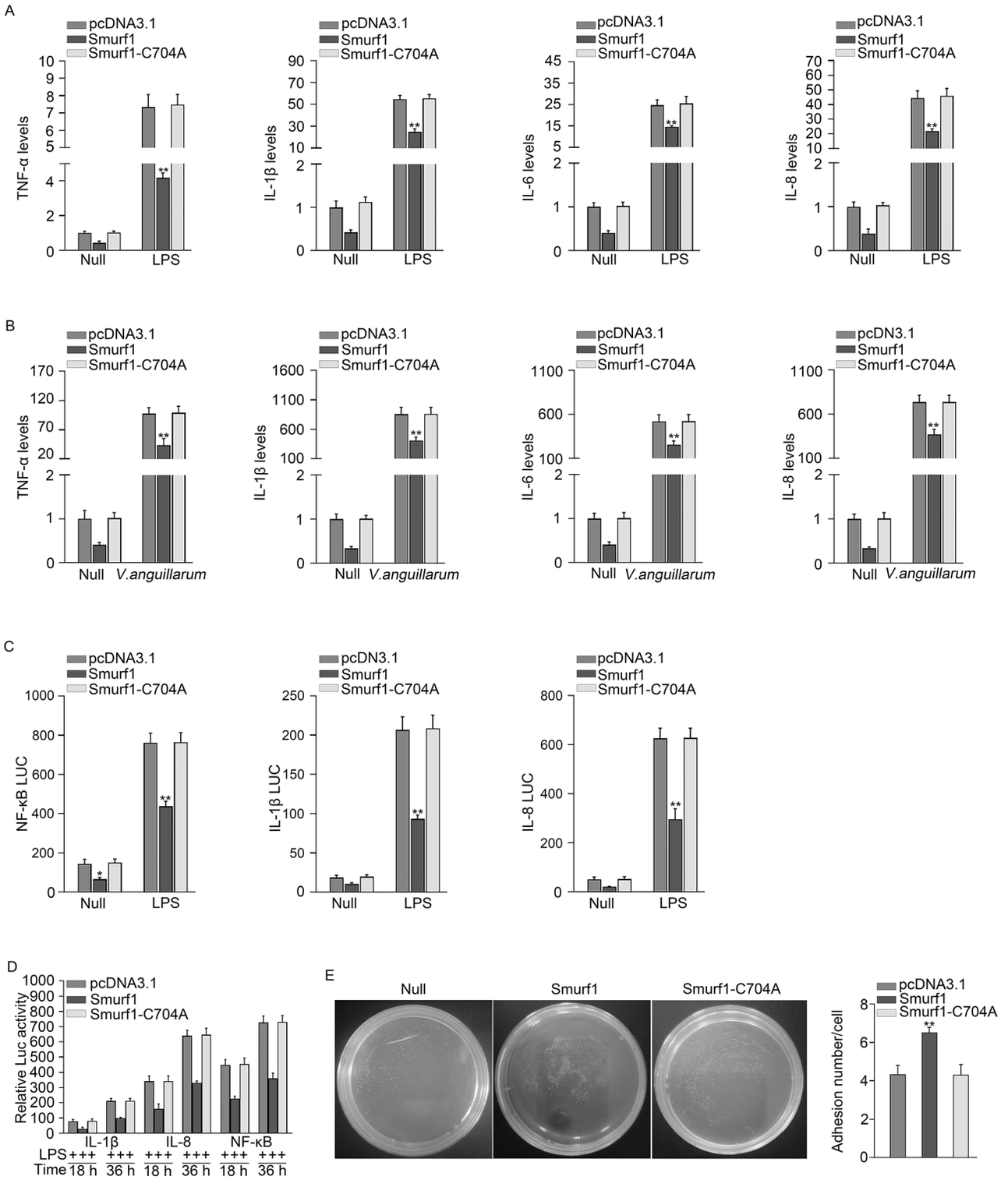


Figure 6 Smurf1-C704A mutant inhibits inflammatory cytokines and the NF-κB signaling pathway

A, B: MKC cells were co-transfected with 0.1 μg of Smurf1-Flag plasmid, 0.1 μg of Smurf1-C704A plasmid, or 0.1 μg of pcDNA3.1. At 36 h post-transfection, cells were treated with LPS for 12 h (A) or infected with *V. anguillarum* for 4 h (B) to induce inflammatory responses. Subsequently, the mRNA levels of TNF-α, IL-1β, IL-6, and IL-8 were determined by qPCR. C, D: EPC cells were co-transfected with the following plasmids: 0.1 μg of Smurf1-Flag plasmid, 0.1 μg of Smurf1-C704A plasmid, or 0.1 μg of pcDNA3.1 empty vector; 0.015 μg of pRL-TK plasmid; and 0.15 μg each of NF-κB, IL-1β, and IL-8 luciferase reporter plasmids. At 36 h post-transfection, cells were treated with LPS for 12 h (C) or at different time points (D). After treatment, the activity of the luciferase reporters was quantified with a dual-luciferase reporter assay and normalized to the Renilla luciferase activity as an internal control. E: MKC cells were transfected with 0.1 μg of Smurf1-Flag plasmid, 0.1 μg of Smurf1-C704A-Flag plasmid, or 0.1 μg of pcDNA3.1 empty vector. After transfection, cells were infected with *V. anguillarum*. At 24 h post-infection, intracellular bacterial counts were determined and expressed as colony-forming units (CFU) per cell. Data are presented as mean±standard error (SE) from at least three independent triplicated experiments. *: $P<0.05$; **: $P<0.01$.

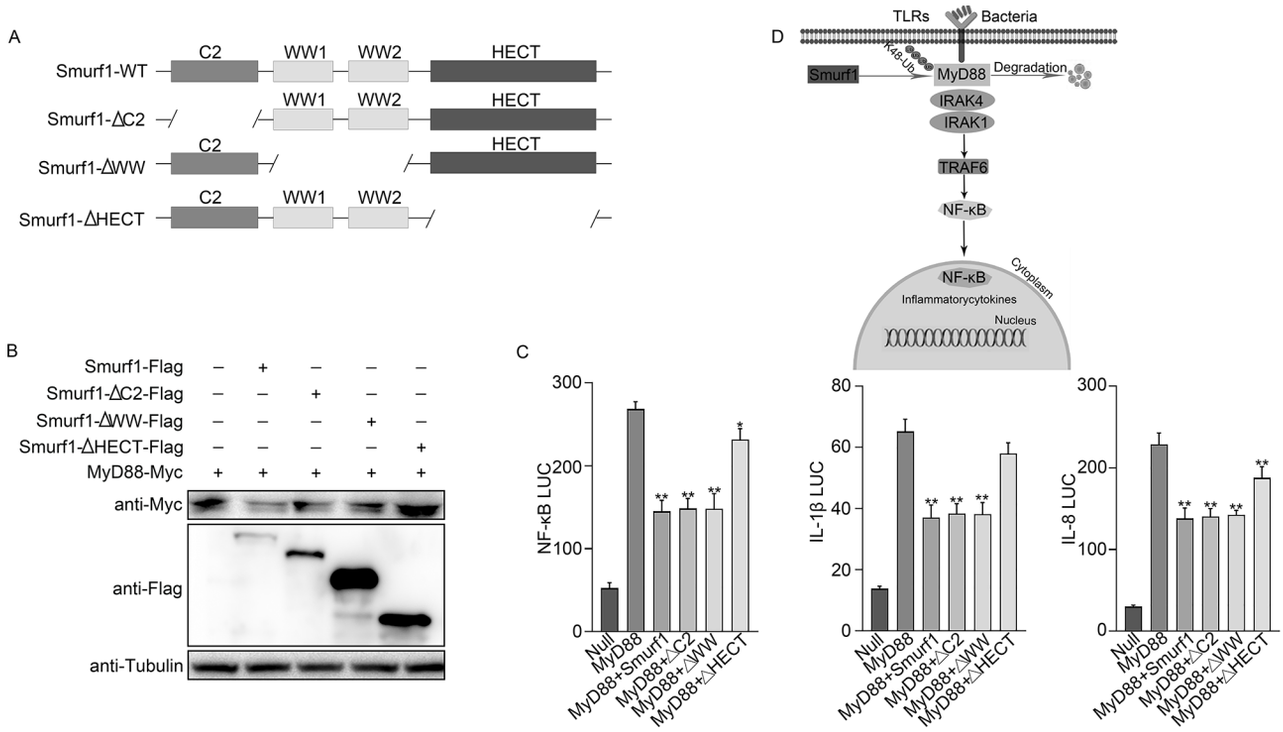


Figure 7 The HECT domain of Smurf1 is critical for the degradation of MyD88

A: Schematic representation of a series of deletion mutant constructs of Smurf1. B: EPC cells were co-transfected with 0.1 μg MyD88-Myc plasmid and either 0.1 μg pcDNA3.1 (empty control), 0.1 μg Smurf1-Flag plasmid, or 0.1 μg of each Smurf1 deletion mutant plasmid (Smurf1-ΔC2-Flag, Smurf1-ΔWW-Flag, Smurf1-ΔHECT-Flag). At 48 h post-transfection, cell lysates were subjected to immunoblot (IB) analysis using primary antibodies: anti-Myc, anti-Flag, and anti-Tubulin. C: EPC cells were co-transfected with the following plasmids: 0.1 μg MyD88-Myc plasmid, 0.015 μg pRL-TK plasmid, 0.15 μg each of NF-κB, IL-1β, and IL-8 luciferase reporter plasmids, and either 0.1 μg pcDNA3.1, 0.1 μg Smurf1-Flag plasmid, or 0.1 μg of each Smurf1 deletion mutant plasmid (Smurf1-ΔC2-Flag, Smurf1-ΔWW-Flag, Smurf1-ΔHECT-Flag). At 48 h post-transfection, the activity of the luciferase reporters was measured using a dual-luciferase reporter assay. Luciferase activity was normalized to Renilla luciferase activity to correct for transfection variability. Data are presented as mean±standard error (SE) from at least three independent triplicated experiments. D: The TLR signaling pathway is activated following bacterial infection. Smurf1 degrades MyD88 and represses MyD88-mediated antibacterial responses. *: P<0.05; **: P<0.01.

DISCUSSION

Our study identifies that in *M. miiuy*, the E3 ubiquitin ligase Smurf1 mediates a post-translational regulatory mechanism by directly targeting the TLR adaptor MyD88 for K48-linked polyubiquitination and proteasomal degradation, thereby negatively regulating NF-κB signaling and antibacterial immunity. Consequently, Smurf1 functions as a key negative regulator that balances antibacterial immunity and inflammatory homeostasis. Innate immune systems require precise regulation of signaling pathways to balance effective pathogen clearance against excessive inflammation. NF-κB is a key transcription factor that plays a central role in regulating innate immunity, development, and cellular survival by driving the expression of inflammatory cytokines. However, its overactivation can lead to tissue damage and pathological inflammation (Burt et al., 2024; Yu et al., 2009). Therefore, precise regulation of NF-κB signaling is critical for maintaining immune homeostasis. Extensive research has been devoted to elucidating the complex regulatory mechanisms underlying NF-κB signaling. For instance, proinflammatory cytokines such as TNFα and IL-6, can induce the expression of TRIM52, whereas TRIM38 which suppresses TNF-α and IL-1β-induced NF-κB activation by regulating the lysosome-dependent degradation of TAB2/3 (Fan et al., 2017; Hu et al., 2014). These findings collectively demonstrate that NF-κB signaling relies on post-translational modifications to ensure precise immune responses, with protein ubiquitination having emerged as a key regulatory mechanism of NF-κB signaling (Hunter, 2007; Liu & Chen, 2011; Sun, 2012). Building on this, research has increasingly focused on the precise regulation of key signaling molecules. In mammals, downregulation of RNF138 drives the progression from colitis to colon cancer via NF-κB signaling, whereas C-Cbl negatively regulates TRAF6-mediated NF-κB activation by promoting K48-linked polyubiquitination of TRAF6 (Jang et al., 2019; Lu et al., 2022). In teleost fish, studies on protein ubiquitination have also begun to reveal complex regulatory mechanisms. For example, IRF3 and IRF9 negatively regulate TLR-mediated NF-κB signaling by targeting TRIF for ubiquitination and degradation (Zhao et al., 2017, 2018a), and Crlf3 mediates K48-linked ubiquitination and subsequent proteasomal degradation of TBK1 (Yan et al., 2023).

As a central hub of innate immunity, MyD88 orchestrates signals from pathogen-associated molecular patterns (PAMPs) to activate NF-κB (Bayer & Alcaide, 2021). Thus, activation of the MyD88-NF-κB pathway culminates in antimicrobial responses that are essential for host defense. However, its dysregulation can provoke excessive inflammation and overproduction of pro-inflammatory cytokines, which are detrimental to host health and can precipitate pathological damage (Gurung et al., 2017). Given this critical balance, tight control of MyD88 protein stability

through PTMs is essential. Accordingly, several E3 ubiquitin ligases, such as the SPOP complex, Cbl-b, and Nrdp1 have been identified as regulators that target MyD88 for ubiquitination (Guillamot et al., 2019; Han et al., 2010; Lee et al., 2011; Li et al., 2020; Meng et al., 2021). For instance, SPOP negatively regulates MyD88-induced NF-κB signaling by mediating K48-linked ubiquitination of MyD88, thereby promoting its proteasomal degradation. Conversely, deubiquitinating enzymes fine-tune this process: CYLD and OTUD4 remove K63-linked ubiquitin chains from MyD88 to attenuate TLR signaling, whereas USP7 cleaves K48-linked chains to stabilize MyD88 and suppress downstream signaling (Lee et al., 2016; Zhang et al., 2022; Zhao et al., 2018b). Although one study showed that *Mycobacterium* PPE36 suppresses host inflammation by recruiting Smurf1 to degrade MyD88, this mechanism depends on an exogenous pathogen effector (Peng et al., 2022). Considering that E3 ligases can target the same substrate through diverse mechanisms and that TLR-NF-κB signaling in aquatic vertebrates differs from that in mammals, the function of E3 ligases in regulating MyD88 in fish deserves further exploration. Our study addresses this gap by demonstrating that Smurf1 in *M. miiuy* acts as a direct and functional E3 ubiquitin ligase for MyD88, acting in a manner dependent on its intrinsic catalytic activity.

Ubiquitination can be categorized into seven distinct types based on the specific lysine residues (K) within ubiquitin (Ub) that are involved in polyubiquitin chain assembly: K6, K11, K27, K29, K33, K48, and K63 (Yau & Rape, 2016). Among these, K48- and K63-linked ubiquitination, which are two subtypes that have received predominant research attention, exhibit distinct functional roles: K48-linked ubiquitination serves as a canonical signal for proteasomal recognition and substrate degradation, whereas K63-linked ubiquitination principally modulates target protein stability and function through non-proteolytic signaling mechanisms. This study provides the first comprehensive elucidation of the mechanism by which Smurf1 regulates immune signaling via K48-linked ubiquitination in teleost fish. Extensive research has established the critical role of Smurf1 in modulating innate immune signaling. For instance, one study reported that TRIB3 drives vascular calcification in chronic kidney disease through Smurf1 self-ubiquitination and dissociation (Li et al., 2025). In HEK293 cells infected with VSV, overexpression of Smurf1 enhanced viral replication, an effect suppressed by the deubiquitinase USP25 (Qian et al., 2018). Recent studies have also shown that teleost Smurf1 can regulate antiviral immunity by degrading TRAF6 (Wang et al., 2024). Extending this functional conservation to antibacterial immunity, our study demonstrates that *M. miiuy* Smurf1 targets the key TLR pathway adaptor protein MyD88. A direct physical interaction between Smurf1 and MyD88 was observed in both HEK293 and EPC cells, indicating cell type independence (Figure 3A, B). Smurf1 promoted the degradation of MyD88 in a dose- and time-dependent manner (Figure 3C, D). This degradation was completely blocked by the proteasome inhibitor MG132 (Figure 4F, G), indicating its dependence on the UPS. Furthermore, Smurf1 specifically enhanced K48-linked polyubiquitination of MyD88 (Figure 5C–H). Ubiquitination assays using ubiquitin constructs containing K48O or K48R revealed that Smurf1 failed to induce MyD88 ubiquitination in the presence of K48R, confirming the K48-linkage specificity of this ubiquitination. K48-linked ubiquitination is a canonical signal for proteasomal degradation (Yau & Rape, 2016). Critically, the E3 ubiquitin ligase activity of Smurf1 is essential for this regulatory process. The HECT domain of Smurf1 contains a conserved catalytic cysteine residue (C704), which is crucial for thioester bond formation with ubiquitin. Smurf1-C704A completely abolished Smurf1-mediated K48-linked ubiquitination of MyD88 (Figure 5H) and restored MyD88 protein levels (Figure 5E, F). Domain deletion experiments further confirmed the paramount importance of the HECT domain itself for Smurf1-mediated MyD88 degradation: Smurf1 lacking the HECT domain (Smurf1-ΔHECT) failed to reduce MyD88 protein abundance (Figure 7B) and could not inhibit NF-κB, IL-1β, and IL-8 reporter gene activity (Figure 7C). In contrast, deletion of the C2 or WW domains had no significant effect on this process. These data rule out a non-catalytic role for Smurf1 in MyD88 regulation and establish the HECT domain as both the catalytic core and the structural basis for this pathway.

Pathogens can exploit the host ubiquitin-proteasome system (UPS) to evade immune responses. To date, research on the prevention and control of pathogenic bacteria in aquaculture remains relatively limited, and various infection prevention strategies are still incomplete, particularly for *V. anguillarum*, where in-depth molecular mechanisms supporting its control are lacking. This study is the first to demonstrate that Smurf1 may be hijacked by *V. anguillarum* to facilitate infection (Figure 1A, B). Smurf1 functions as a negative regulator to suppress excessive inflammation; however, during pathogenic infection, its upregulated activity is specifically hijacked by the bacterium, thereby impairing the host's immune clearance capacity and ultimately promoting infection of the host. To clarify Smurf1's function, we explored its role in bacterial-induced inflammation: Smurf1 inhibits proinflammatory cytokine induction by mediating MyD88 degradation. Functional experiments confirmed this regulation: Smurf1 knockdown reduced *V. anguillarum* proliferation and adhesion (Figure 1D–F), while its overexpression enhanced bacterial adhesion, an effect dependent on its E3 ubiquitin ligase activity (Figure 6E). Mechanistically, Smurf1 targets MyD88 ubiquitination, leading to MyD88 degradation via the host UPS. This inhibition of downstream antibacterial factor production helps *V. anguillarum* hijack the host's innate immunity to evade clearance. However, key questions remain regarding the specific regulatory roles of amino acid residues in Smurf1's HECT domain, whether other pathogens also exploit the Smurf1-MyD88-UPS pathway for immune escape, and the need to further validate Smurf1's role in antibacterial immunity through in vivo experimentation. In conclusion, as a key E3 ubiquitin ligase, Smurf1 can directly bind to MyD88 and promote the ubiquitination-mediated degradation of MyD88 via the UPS. This process not only regulates the host's antibacterial immune response against *V. anguillarum* but also suppresses excessive inflammatory responses to reduce tissue damage, thereby highlighting the central role of Smurf1 in coordinating antibacterial defense and inflammatory homeostasis in fish.

SUPPLEMENTARY DATA

Supplementary data to this article can be found online.

COMPETING INTERESTS

The authors declare that they have no competing interests.

AUTHORS' CONTRIBUTIONS

W.D.: Writing—original draft, Investigation, Methodology, Formal analysis. T.X. and Y.S.: Writing—review & editing, Conceptualization, Resources,

Supervision. All authors read and approved the final version of the manuscript.

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