Smurf1 Protein Negatively Regulates Interferon-γ Signaling through Promoting STAT1 Protein Ubiquitination and Degradation*

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Background: IFN signaling must be tightly regulated.
Results: Smurf1 ubiquitinates and degrades STAT1.
Conclusion: Smurf1 negatively regulates IFN-γ signaling.
Significance: Identification of Smurf1 as a new feedback negative regulator of IFN-γ signaling may make Smurf1 a valuable target in the design of therapeutic drugs.

Interferons are important cytokines that mediate antiviral, antiproliferative, antitumor, and immunoregulatory activities. However, uncontrolled IFN signaling may lead to autoimmune diseases. Here we identified Smurf1 as a negative regulator for IFN-γ signaling by targeting STAT1 for ubiquitination and proteasomal degradation. Smurf1 interacted with STAT1 through the WW domains of Smurf1 and the PY motif in STAT1 and catalyzed K48-linked polyubiquitination of STAT1. Interestingly, the Smurf1-mediated ubiquitination and degradation did not require STAT1 tyrosine and serine phosphorylation. Subsequently, overexpression of Smurf1 in cells led to enhanced IFN-γ-mediated STAT1 activation and antiviral immune responses, whereas knockdown of Smurf1 enhanced IFN-γ-mediated STAT1 activation, expression of STAT1 target genes, and antiviral immune responses. Furthermore, IFN-γ stimulation led to enhanced expression of Smurf1. Therefore, our results demonstrate that Smurf1 is a negative feedback regulator for IFN-γ signaling by targeting STAT1 for ubiquitination and proteasomal degradation.

The HECT-type E3 ligase Smad ubiquitination regulation factor 1 (Smurf1) was originally identified as the E3 ligase of Smad1/5, and it dorsalizes ventral mesoder and neuralizes ectoderm by modulating BMP signaling in Xenopus embryo (1). Smurf1 plays a critical role in the regulation of embryonic development, cell polarity, and bone homeostasis by targeting the degradation of Smad1/5, TGF-βR, RhoA, MEKK2, Prickle 1, and JunB (1–6). Although the functions of Smurf1 in the regulation of TGF-β and BMP signaling are well defined, other cellular signaling pathways, especially in the immune responses regulated by Smurf1, are not clear. In this regard, two recent studies have demonstrated that Smurf1 can regulate immune response through mediating ubiquitination and proteasomal degradation of TRAFs and MyD88, respectively (7, 8).

Interferons (IFNs) are important cytokines that play essential roles in antiviral, antiproliferative, and antitumor activities (9, 10). IFN primarily signals through the Jak-STAT1 pathway leading to the activation of STAT1 and subsequent transcription of STAT1 target genes (11). The importance of STAT1 in IFN-γ and IFN-α/β signaling was clearly established by studies using mutant cell lines and the generation of STAT1-deficient mice. STAT1 knock-out mice show high susceptibility to microbial and viral infections and tumor formation due to the abrogation of the induction of several well known IFN-inducible genes (12, 13). As an essential molecule in IFN signaling, STAT1 has been reported to be controlled by post-translational modifications including phosphorylation, acetylation, and ubiquitination (14, 15).

In the present study, we identified Smurf1 as a negative feedback regulator for IFN-γ signaling by targeting STAT1 for ubiquitination and proteasomal degradation. Smurf1 interacted with STAT1 through the WW domains of Smurf1 and the PY motif in STAT1. Further, Smurf1 promoted K48-linked polyubiquitination and proteasomal degradation of STAT1. Subsequently, overexpression of Smurf1 attenuated IFN-γ-induced STAT1 activation and antiviral immune responses, whereas knockdown of Smurf1 greatly increased IFN-γ-induced STAT1 activation, expression of STAT1 target genes, and antiviral immune responses.

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2 The abbreviations used are: Smurf1, Smad ubiquitination regulation factor 1; GAS, IFN-γ-activated site; iNOS, inducible nitric oxide synthase; Ub, ubiquitin; VSV, vesicular stomatitis virus; BMP, bone morphogenetic protein; TRAF, TNF receptor-associated factor; CA, catalytically inactive.

3 Throughout this study, K48 and K63 indicate ubiquitin mutant vectors that contain arginine substitutions of all of its lysine residues except the one at positions 48 and 63, respectively.
EXPERIMENTAL PROCEDURES

Mice and Cells—C57BL/6J mice were obtained from Joint Ventures Sipper BK Experimental Animal (Shanghai, China). All animal experiments were undertaken in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals, with the approval of the Scientific Investigation Board of the Medical School of Shandong University, Jinan, Shandong Province, China. Mouse macrophage cell line RAW264.7, human HEK293 cells, and mouse NIH3T3 cells were obtained from the American Type Culture Collection (Manassas, VA). Mouse primary peritoneal macrophages were prepared as described (16). The cells were cultured at 37 °C under 5% CO₂ in DMEM supplemented with 10% FBS (Invitrogen-Invitrogen), 100 units/ml penicillin, and 100 μg/ml streptomycin.

Reagents—N-Carbobenzoxyl-l-leucinyl-l-norleucin (MG132), monoclonal anti-FLAG M2 antibody, and anti-FLAG M2 affinity gel were purchased from Sigma-Aldrich. The antibodies specific for STAT1 p84/p91, Smurf1, HA, Ub, β-actin, and protein G-agarose used for immunoprecipitation were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). The antibodies specific to Myc, phospho-STAT1 at Tyr-701, and phospho-STAT1 at Ser-727 were from Cell Signaling Technology (Beverly, MA). Anti-GAPDH was purchased from Abcam. Mouse primary peritoneal macrophages were provided by Dr. Yui Xiao (Institute Pasteur of Shanghai, Chinese Academy of Sciences, China). FLAG-tagged Smurf1 truncations were gifts from Dr. Steven Brand (University of California, San Francisco, CA). Mouse primary peritoneal macrophages were prepared as described (16). The cells were cultured at 37 °C under 5% CO₂ in DMEM supplemented with 10% FBS (Invitrogen-Invitrogen), 100 units/ml penicillin, and 100 μg/ml streptomycin.

Plasmid Constructs and Transfection—Mammalian expression plasmids for FLAG-tagged STAT1 and luciferase reporter constructs bearing the mouse iNOS promoter have been described previously (17). Myc-tagged STAT1 and Smurf1 were obtained by PCR and cloned into the pCMV-Myc (Promega). FLAG-STAT1-Y701F, and FLAG-STAT1-S727A were generated using the KOD-Plus-Mutagenesis kit (Toyobo, Osaka, Japan). All constructs were confirmed by DNA sequencing. GAS-Luc reporter plasmids were purchased from Stratagene. FLAG-Smurfl WT and FLAG-Smurfl CA mutant (the catalytically inactive form of Smurf1) were from Jeffrey L. Wrana (Mount Sinai Hospital, Canada). FLAG-tagged Smurf1 truncations were gifts from Dr. Hong-Rui Wang (Xiamen University, China). Expression vectors for HA-Ub WT and mutants K48 and K63 were from Dr. Hui Xiao (Institute Pasteur of Shanghai, Chinese Academy of Science, China). For transient transfection of plasmids into RAW264.7 cells, jetPEI reagents were used (Polyplus-transfection). For transient silencing, duplexes of small interfering RNA were transfected into cells with the GenePORTER 2 transfection reagent (Genlanits, San Diego, CA) according to the standard protocol as described (18). The target sequence for transient silencing was 5′-GGAGGAAGGUUGACAUUATT-3′ (antisense) for Smurf1, and the “scrambled” control sequence was 5′-UUCUCGGGAGCUCAGCUGT-3′.

Assay of NO Production—NO released into the culture medium was measured indirectly as hydrolyzed NO derivatives (NOx: nitrites and nitrates) using the Griess reagent kit (Dojindo Molecular Technologies, Kumamoto, Japan), in accordance with the manufacturer’s instructions. The amount of culture medium per well was normalized to the total cell number in individual samples. The NOx concentration in the medium was determined by measuring absorbance at 540 nm and using a standard curve.

RNA Quantification—Total RNA was extracted with TRIzol reagent according to the manufacturer’s instructions (Invitrogen). Specific primers used for RT-PCR assays were 5′-AC-TCCCTGTACGCCCTCAGGAACTT-3′ (sense), 5′-GT-CCTCTGCACACTGGTTCCTTGTGCTATA-3′ (antisense) for Smurf1, and 5′-CAACTACATGGTCTACATGGTC-3′ (sense), 5′-CACCAGTACCTACCAAGC-3′ (antisense) for GAPDH. Specific primers for Cxcl9, Cxcl10, IRF1, and iNOS were used as described (19).

Coimmunoprecipitation and Immunoblotting Analysis—To detect interaction between endogenous Smurf1 and STAT1, whole-cell extracts were prepared by lysing the cells in immunoprecipitation buffer containing 1% (w/v) Nonidet P-40, 50 mM Tris-HCl, pH 7.4, 50 mM EDTA, 150 mM NaCl, and a protease inhibitor mixture (Merck). After centrifugation for 10 min at 14,000 × g, supernatants were collected and incubated with protein G Plus-agarose immunoprecipitation reagent (Santa Cruz Biotechnology) together with anti-Smurfl antibody. After 6 h of incubation, beads were washed five times with immunoprecipitation buffer. Immunoprecipitates were eluted by boiling with 1% (w/v) SDS sample buffer. To detect interaction between exogenous Smurf1 and STAT1, lysates from HEK293 cells transfected with STAT1 or its mutants and Smurf1 or its mutants were incubated with anti-FLAG M2 affinity gel for 3 h. After washing three times with TBS buffer, immunoprecipitates were separated from the beads by adding 3×FLAG peptide elution solution and then boiled and subjected to SDS-PAGE. For immunoblotting analysis, immunoprecipitates or whole-cell lysates were loaded and subjected to SDS-PAGE, transferred onto PVDF membranes, and then blotted as described previously (16).

Assay of Luciferase Activity—Luciferase activity was measured with the Dual-Luciferase reporter assay system according to the manufacturer’s instructions (Promega) as described. Data were normalized for transfection efficiency by division of firefly luciferase activity with that of Renilla luciferase.

In Vivo Ubiquitination Assays—To measure STAT1 ubiquitination in vivo, HEK293 cells were transiently transfected with HA-ubiquitin (HA-Ub), FLAG-STAT1, Myc-Smurfl wild-type (WT), and Myc-Smurfl E3-inactive mutant (C699A, CA), as indicated. 24 h after transfection, cells were harvested under denaturing conditions in buffer containing 50 mM Tris (pH 7.4), 140 mM NaCl, 1% SDS. Samples were boiled for 5 min and then diluted 10-fold with buffer containing 50 mM Tris (pH 7.4), 140 mM NaCl, 1% Triton X-100, and protease inhibitor mixture (Merck). Samples were centrifuged at 16,000 × g for 10 min. Cell lysates were then incubated with anti-FLAG M2 affinity gel for 3 h. After washing three times with TBS buffer, immunoprecipitates were separated from the beads by adding 3×FLAG peptide elution solution and then boiled and subjected to SDS-PAGE. Immunoblot analysis was subsequently performed using the indicated antibodies.
VSV Plaque Assay and Detection of Virus Replication—Vesicular stomatitis virus (VSV) plaque assay was performed as described (20). The HEK293 cells ($2 \times 10^5$) were transfected with the indicated plasmids for 24 h following treatment with recombinant human IFN-γ (10 ng/ml) for 8 h. At 1 h after VSV infection, cells were washed with PBS for three times, and then medium was added. The supernatants were harvested at 24 h after washing. The supernatants were diluted from 1:10³ to 1:10⁶ and then used to infect confluent HEK293 cells cultured on 24-well plates. At 1 h after infection, the supernatant was removed, and 1% methylcellulose was overlaid. At 2 days after infection, overlay was removed; cells were fixed with 4% form-
aldehyde for 20 min and stained with 0.1% crystal violet. Plaques were counted, averaged, and multiplied by the dilution factor to determine viral titer as pfu/ml. Total HEK293 cellular RNA was extracted at 18 h after VSV infection, and VSV RNA replicates were examined by quantitative RT-PCR as described (21). Primers for VSV were as follows: 5′/H11032-ACGGCGTACTTC-

FIGURE 2. Smurf1 interacts with STAT1. A, Myc-Smurfl (M-Smurfl) and FLAG-STAT1 (F-STAT1) were cotransfected into HEK293 cells, and the lysates from transfected cells were subjected to immunoprecipitation (IP) with anti-FLAG antibody followed by immunoblotting (IB) analysis with anti-Myc antibody. B, Myc-STAT1 was cotransfected into HEK293 with FLAG-Smurfl(WT) or FLAG-Smurfl(CA), and cells were lysed and immunoprecipitated with anti-FLAG antibody followed by immunoblotting analysis with anti-Myc antibody. C, RAW264.7 cells were treated with IFN-γ (10 ng/ml) for the indicated times, and cell lysates were subjected to immunoprecipitation with anti-Smurfl antibody followed by immunoblotting analysis with anti-STAT1 antibody. D, left panel, HEK293 cells were transiently transfected with combinations of Myc-STAT1 and FLAG-Smurfl or its mutants as indicated, and cell lysates were subjected to immunoprecipitation using anti-FLAG antibody followed by immunoblotting analysis with anti-Myc antibody. Right panel, schematic description of Smurf1 mutants and interaction with STAT1. E, Myc-Smurfl and FLAG-STAT1(WT) or FLAG-STAT1(PY/AA) were cotransfected into HEK293 cells, and cell lysates were subjected to immunoprecipitation using anti-FLAG antibody followed by immunoblotting analysis with anti-Myc antibody. Similar results were obtained with three independent experiments.

FIGURE 1. Smurf1 negatively regulates IFN-γ signaling. A, RAW264.7 were transfected with GAS reporter plasmid together with Smurf1 expression plasmid or control (Ctrl) plasmid, and luciferase activity was measured after treatment with IFN-γ (10 ng/ml) for 12 h. Data are shown as mean ± S.D. (n = 6) of one representative experiment. B, RAW264.7 were transfected with GAS reporter plasmid together with increasing amounts of Smurf1 expression plasmid or control plasmid, and luciferase activity was measured after treatment with IFN-γ (10 ng/ml) for 12 h. Data are shown as mean ± S.D. (n = 6) of one representative experiment. C, RAW264.7 were transfected with GAS reporter plasmid together with increasing amounts of Smurf1 expression plasmid or control plasmid, and luciferase activity was measured after treatment with IFN-γ (10 ng/ml) for 12 h. Data are shown as mean ± S.D. (n = 6) of one representative experiment. D, mouse peritoneal macrophages were transfected with siRNA (siNc) or Smurf1 siRNA (siSmurf1) for 48 h, and Smurf1 mRNA expression and protein expression were measured with quantitative RT-PCR and Western blotting (IB) analysis, respectively. E, mouse peritoneal macrophages treated as in D were stimulated with IFN-γ (10 ng/ml) for various times. Expression of iNOS mRNA and protein expression was measured with quantitative RT-PCR and Western blotting (IB) analysis, respectively. F, mouse peritoneal macrophages treated as in D were stimulated with IFN-β (20 ng/ml) for various times. Expression of iNOS mRNA was measured with quantitative RT-PCR. G, mouse peritoneal macrophages treated as in D were stimulated with IFN-γ (100 ng/ml) for various times. Nitrite production in the supernatants was assayed using the Griess reagent kit. *, p < 0.05, **, p < 0.01, and ***, p < 0.001 (Student’s t test). Data are one representative of at least three independent experiments.
Smurf1 Ubiquitinates STAT1

CAGATGG-3' (sense) and 5'-CTCGGTCAAGATCCAGTTGGT-3' (antisense).

Results

Smurf1 Negatively Regulates IFN-γ Signaling—Smurf1 plays a critical role in the regulation of TGF-β signaling and BMP signaling by targeting the degradation of several target proteins (22). To explore the functions of Smurf1 in other signaling pathways, we initially investigated the effect of Smurf1 on interferon signaling. The binding of IFN-γ to its receptor, interferon-γ receptor (IFNGR), results in the phosphorylation of STAT1 and formation of STAT1 homodimers named γ-interferon activation factor (GAF). γ-Interferon activation factor migrates to the nucleus and binds to a DNA element termed IFN-γ-activated site (GAS), resulting in the activation of transcription and induction of STAT1 target genes (23). To investigate the role of Smurf1 in IFN-γ signaling, the effect of Smurf1 on the activation of the STAT1 cis-reporting plasmid GAS-Luc was investigated. As shown in Fig. 1A, overexpression of Smurf1 significantly inhibited IFN-γ-induced transcriptional activation of GAS-Luc reporter plasmids. Furthermore, Smurf1 inhibited IFN-γ-induced activation of GAS-Luc reporter in a dose-dependent manner (Fig. 1B). Similarly, Smurf1 also significantly inhibited IFN-γ-induced transcriptional activation of iNOS gene promoter in a dose-dependent manner (Fig. 1C).

To directly examine the function of Smurf1 in IFN-γ-mediated gene activation, mouse peritoneal macrophages were transfected with control siRNA or Smurf1 siRNA to silence Smurf1 expression, and then the cells were treated with IFN-γ for various times. Both Smurf1 mRNA and protein were substantially decreased after transfection of Smurf1 siRNA when compared with control siRNA transfection (Fig. 1D). IFN-γ-induced expression of Cxcl9 (CXC chemokine ligand 9), Cxcl10 (CXC chemokine ligand 10), Irf1 (interferon regulatory factor 1), and Nos2 (inducible nitric oxide synthase) was significantly enhanced in Smurf1 siRNA-transfected macrophages as measured by quantitative real-time PCR (Fig. 1E). Similarly, IFN-β-induced expression of Cxcl9, Cxcl10, Ly6e, and Irf203 was also significantly enhanced in Smurf1 siRNA-transfected macrophages (Fig. 1F). Accordingly, IFN-γ-induced NO production was significantly increased in Smurf1 siRNA-transfected macrophages (Fig. 1G). Altogether, these results suggest that Smurf1 negatively regulates IFN signaling.

Smurf1 Physically Interacts with STAT1—STAT1 plays an essential role in IFN-γ signaling. Furthermore, STAT1 has been reported to interact with Smurf1 in a large scale luminescence-based mammalian interactome mapping (24). Thus, we hypothesized that Smurf1 negatively regulates IFN-γ signaling by targeting STAT1. To confirm the interaction between Smurf1 and STAT1, Myc-Smurf1 and FLAG-STAT1 were cotransfected into HEK293 cells followed by coimmunoprecipitation with FLAG antibody and Western blotting with Myc antibody. As shown in Fig. 2A, Myc-smurf1 was coimmunoprecipitated with FLAG-STAT1 (Fig. 2B). Similarly, Myc-STAT1 was also coimmunoprecipitated with FLAG-Smurf1 CA mutant (Fig. 2B), indicating that Smurf1 interacts with STAT1 independent of its E3 ligase activity. Interaction of endogenous proteins was confirmed in macrophages stimu-
lated with IFN-γ for various times with coimmunoprecipitation assays and Western blotting. STAT1 was coimmunoprecipitated with Smurf1 in both IFN-γ-simulated and unstimulated conditions (Fig. 2C). As a control, the interaction could not be detected with normal IgG.

It has been reported that Smurf1 interacts with its target proteins through the WW domains of Smurf1 and the proline-rich (PPXY) motif in the substrate proteins (25). To map the Smurf1 domains required for the interaction with STAT1, various Smurf1 mutants were cotransfected into HEK293 cells together with FLAG-STAT1 and Myc-Smurf1 or control vector. STAT1 ubiquitination was detected by immunoprecipitation with anti-FLAG antibody and immunoblotting with anti-HA antibody. As a control, the interaction could not be detected with normal IgG.

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Smurf1 Ubiquitinates STAT1

tigate the function of Smurf1 on the degradation of STAT1. An increasing concentration of Smurf1 expression plasmid was cotransfected into HEK293 cells together with a constant concentration of STAT1 expression plasmid. As shown in Fig. 3A, STAT1 protein expression was substantially decreased with transfection of Smurf1 expression plasmid. In sharp contrast, Smurf1 CA mutant could not decrease the STAT1 protein level, indicating that the E3 ligase activity of Smurf1 is required for the degradation of STAT1. Similarly, Smurf1 transfection could decrease the level of endogenous STAT1 protein (Fig. 3B). To determine whether Smurf1 inhibits STAT1 protein expression under physiological conditions, Smurf1 siRNA was transfected into mouse primary peritoneal macrophages, and STAT1 protein expression was measured. Transfection of Smurf1 siRNA significantly increased total STAT1 protein level in macrophages when compared with control siRNA-transfected cells (Fig. 3C). Accordingly, IFN-γ-induced STAT1 phosphorylation was also significantly increased (Fig. 3C). These data suggest that Smurf1 promotes the degradation of STAT1.

Smurf1 Promotes K48-linked Polyubiquitination of STAT1—Because Smurf1 is an E3 ubiquitin ligase and causes STAT1 degradation, STAT1 ubiquitination mediated by Smurf1 was investigated. Expression plasmids for FLAG-STAT1 and HA-ubiquitin were transfected into HEK293 cells together with Myc-Smurf1, Myc-Smurf1 CA, or control plasmid followed by immunoprecipitation and Western blot analysis of STAT1 ubiquitination. As shown in Fig. 4A, STAT1 polyubiquitination was greatly increased in the presence of Smurf1 (lane 3) when compared with control vector (lane 2). Importantly, transfection of Smurf1 CA mutant could not increase STAT1 polyubiquitination (lane 4). To determine the form of ubiquitin chains linked to STAT1, ubiquitin mutant vectors K48 and K63, were used in transfection assays. Immunoprecipitation and Western blotting analysis showed that Smurf1 induced K48-linked polyubiquitination of STAT1 (lanes 2 and 4), but not K63-linked polyubiquitination (Fig. 4B, lane 6). K48-linked protein ubiquitination leads to the degradation of the corresponding protein by 26 S proteasome (26). Consistently, Smurf1-induced degradation of STAT1 protein could be reversed by proteasome inhibitor MG132 (Fig. 4C). Taken together, these results indicate that Smurf1 promotes K48-linked polyubiquitination and subsequent proteasomal degradation of STAT1.

STAT1 Phosphorylation Is Not Required for Smurf1-mediated STAT1 Degradation—Because STAT1 must be phosphorylated on tyrosine 701 and serine 727 to be fully active in transcription (27), we investigated whether STAT1 degradation mediated by Smurf1 requires phosphorylation. Expression plasmids for STAT1 wild-type or mutants (Y701F or S727A) were transfected into HEK293 together with increasing amounts of Smurf1, and the level of STAT1 proteins was measured with Western blotting. As shown in Fig. 5A, Smurf1 could induce the degradation of STAT1 WT and STAT1 mutants Y701F and S727A. Consistently, Smurf1 could promote polyubiquitination of both STAT1 WT and STAT1 mutants Y701F and S727A (Fig. 5B). These data indicated that the degradation of STAT1 mediated by Smurf1 is independent of tyrosine and serine phosphorylation.

Smurf1 Negatively Regulate Antiviral Immune Responses—Previous studies showed that full complementation of the antiviral state requires full-length STAT1 (28). Because Smurf1 is a physiological suppressor of IFN-γ signaling, we speculate that Smurf1 may play a role in the cellular antiviral response. To directly investigate the effect of Smurf1 on IFN-γ-mediated antiviral responses, VSV was used to infect HEK293 cells and macrophages. Plaque assay of HEK293 cells infected with VSV showed that overexpression of Smurf1 substantially increased viral replication (Fig. 6A). In sharp contrast, Smurf1 CA mutant, which lost the ability to promote ubiquitination and degradation of STAT1, could not increase viral replication (Fig. 6A). Similarly, VSV RNA replication in HEK293 cells was significantly increased in Smurf1-transfected cells when compared with control vector- or Smurf1 CA-transfected cells as measured by quantitative RT-PCR (Fig. 6B). To further confirm the function of Smurf1 on VSV replication under physiological conditions, Smurf1 expression was silenced by Smurf1 siRNA transfection in mouse peritoneal macrophages, and then the macrophages were stimulated with IFN-γ and infected with VSV. Plaque assay showed that transfection of Smurf1 siRNA greatly decreased VSV viral replication in macrophages (Fig. 6C). Accordingly, knockdown of
Smurf1 significantly decreased intracellular VSV RNA replication when compared with control siRNA-transfected macrophages (Fig. 6D). Taken together, these data demonstrate that Smurf1 negatively regulates cellular antiviral immune responses.

**IFN-γ Induces Expression of Smurf1**—Having established the negative role of Smurf1 in IFN-γ signaling, we finally examined the effect of IFN-γ on Smurf1 expression. RAW264.7 macrophages were treated with IFN-γ for various times, and the level of Smurf1 protein was analyzed with immunoblotting. As shown in Fig. 7A, the expression level of Smurf1 protein was significantly increased and reached peak expression at 8–24 h after IFN-γ stimulation. Surprisingly, IFN-β could not induce Smurf1 expression in RAW264.7 macrophages (Fig. 7B). Smurf1 protein expression was also induced with IFN-γ stimulation in NIH3T3 and HEK293 cells (Fig. 7, C and D). These results suggest that Smurf1 is induced by IFN-γ and acts as a negative feedback regulator for IFN-γ signaling.

**DISCUSSION**

Interferons have antiviral, antiproliferative, and immunoregulatory functions (9, 10). However, uncontrolled IFN signaling may lead to autoimmune diseases (29). Therefore, IFN signaling must be tightly controlled. As an essential molecule downstream of IFN signaling, the importance of STAT1 has been manifested by the construction of STAT1-deficient mice (12). Thus, regulation of STAT1 activation represents a novel pathway to modulate IFN signaling.

In this study, we demonstrated that the E3 ubiquitin ligase Smurf1 promotes STAT1 ubiquitination and degradation to negatively regulate interferon-γ signaling. Using coimmunoprecipitation assays, Smurf1 was found to interact with STAT1. Like most interactions between Smurf1 and its substrate, domain-mapping experiments suggest that the WW domains of Smurf1 are sufficient for its interaction with STAT1. The PY motif in STAT1 directly mediated the interaction with Smurf1. Furthermore, we found that Smurf1 promotes STAT1 ubiquitination and degradation through a proteasome-dependent manner. Consequently, Smurf1 overexpression significantly decreased IFN-γ signaling and antiviral immune responses, whereas knockdown of Smurf1 expression increased STAT1 phosphorylation, expression of STAT1 target genes, and antiviral immune responses.
Importantly, Smurf1 expression is induced by IFN-γ stimulation in several types of cell. Thus, Smurf1 is a negative feedback regulator for IFN-γ signaling by targeting STAT1 ubiquitination and degradation.

Previous studies have demonstrated that ubiquitination and degradation of STAT1 are an important mechanism to down-regulate IFN signaling. Kim and Maniatis (30) were the first to report STAT1 ubiquitination. They found that STAT1 proteins activated by interferon-stimulated response STAT1 ubiquitination. They found that STAT1 proteins activated by interferon-gamma were stabilized by a proteasome inhibitor MG132. Further, they showed that STAT1 was ubiquitinated upon IFN-γ stimulation and that STAT1 phosphorylation was required for its ubiquitination. STAT1 ubiquitination was also detected during virus infection. Some viruses, including SV5 and mumps virus, use their V protein to block IFN signaling by targeting STAT1 for ubiquitination and proteasome mediated degradation (31, 32). However, the E3 ligases responsible for the STAT1 ubiquitination are not defined. In this regard, PDLIM2 has been identified as an E3 ligase for STAT1 ubiquitination (15, 17). PDLIM2, as a nuclear protein, was shown to specifically interact with the tyrosine-phosphorylated STAT1 and mediates the degradation of active STAT1. In this study, we demonstrated that Smurf1 is another E3 ligase involved in STAT1 ubiquitination and degradation. Importantly, we found that STAT1 constitutively interacts with Smurf1 in the cytoplasm. STAT1 Y701F and S727A mutants could be ubiquitinated and degraded by Smurf1 with the same efficiency as the WT STAT1. Thus, our findings suggest that Smurf1 is a cellular ubiquitin ligase with specificity toward unphosphorylated STAT1 proteins.

Smurf1 plays essential roles in development (22). The possible role of the Smurf1 protein in immunity has been reported recently. Smurf1 was demonstrated to mediate the ubiquitination and degradation of MyD88, an essential adaptor molecule in Toll-like receptor and IL-1 signaling (8). Smurf1 has also been reported to target various TRAF family proteins (7). Given the importance of TRAF family proteins in immunity, these data indicate that Smurf1 plays an important role in immune responses. We provided evidence to show that Smurf1 negatively regulates IFN signaling by targeting STAT1 ubiquitination and degradation. Therefore, Smurf1 is a very important immune regulator through promoting degradation of various target proteins.

In conclusion, we demonstrated that the E3 ubiquitin ligase Smurf1 mediated the ubiquitination and degradation of unphosphorylated STAT1 to negatively regulate IFN-γ signaling. Overexpression of Smurf1 attenuated IFN-γ-mediated STAT1 activation and antiviral immune responses, whereas knockdown of Smurf1 greatly increased IFN-γ-induced STAT1 activation, expression of STAT1 target genes, and antiviral immune responses. Thus, Smurf1 may be a valuable target for the design of therapeutic drugs to modulate IFN signaling that are involved in antiviral immunity and autoimmune diseases.

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