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Ndfip1 Regulates Itch Ligase Activity and Airway Inflammation via UbcH7

Mahesh Kathania,* Minghui Zeng,* Viveka Nand Yadav,† Seyed Javad Moghaddam,‡ Baoli Yang,§ and K Venuprasad*†

The ubiquitin-ligating enzyme (E3) Itch plays a crucial role in the regulation of inflammation, and Itch deficiency leads to severe airway inflammation. However, the molecular mechanisms by which Itch function is regulated remain elusive. In this study, we found that nontypeable Haemophilus influenzae induces the association of Itch with Ndfip1. Both Itch−/− and Ndfip1−/− mice exhibited severe airway inflammation in response to nontypeable Haemophilus influenzae, which was associated with elevated expression of proinflammatory cytokines. Ndfip1 enhanced Itch ligase activity and facilitated Itch-mediated Tak1 ubiquitination. Mechanistically, Ndfip1 facilitated recruitment of ubiquitin-conjugating enzyme (E2) UbcH7 to Itch. The N-terminal region of Ndfip1 binds to UbcH7, whereas the PY motif binds to Itch. Hence, Ndfip1 acts as an adaptor for UbcH7 and Itch. Reconstitution of full-length Ndfip1 but not the mutants that fail to interact with either UbcH7 or Itch, restored the defect in Tak1 ubiquitination and inhibited elevated proinflammatory cytokine expression by Ndfip1−/− cells. These results provide new mechanistic insights into how Itch function is regulated during inflammatory signaling, which could be exploited therapeutically in inflammatory diseases. The Journal of Immunology, 2015, 194: 2160–2167.

Inflammation is essential for host defense against invading pathogens, such as viruses and bacteria (1). The inflammatory response must be resolved after the pathogens are cleared, because unchecked inflammation can cause host tissue damage, leading to pathological conditions, such as autoimmunity and malignancy (2). However, the mechanism that controls resolution of the inflammatory response is incompletely understood.

Ubiquitin (Ub)-mediated degradation of signaling intermediates is an important means of termination of the inflammatory response (3). Ubiquitination is achieved via an enzyme cascade consisting of ubiquitin-activating, ubiquitin-conjugating (E2), and ubiquitin-ligating (E3) enzymes (4, 5). Ub is activated by the Ub-activating enzymes and is transferred to E2. E2s, in turn, interact with E3s, which mediate conjugation of Ub to target proteins. There are two classes of E3 ligases: the RING domain class and the HECT domain class. RING domain E3s facilitate the direct transfer of Ub from E2 to a substrate without the formation of a transient covalent E3-Ub thioester intermediate. The HECT domain-containing E3 enzymes act as intermediate acceptors of Ub. The catalytic activity of the HECT domain ubiquitinates the target protein and catalyzes formation of polyubiquitin chains. The E2s catalyze a thioester covalent attachment of Ub to a conserved cysteine residue in the HECT domain, and this Ub is transferred to the target protein (5).

Itch belongs to the HECT family of E3 ligases, which contains a PKC-related C2 domain, four WW domains, and the HECT ligase domain. Deficiency of Itch in mice results in severe multigorgan inflammatory disease, including chronic pulmonary interstitial inflammation and alveolar proteinosis (6, 7). Further, a truncated human Itch mutation results in severe lung inflammation in a group of pediatric patients (8).

Originally, Ndfip1 was identified as a Nedd4-interacting protein (9). It is composed of three highly conserved transmembrane domains and cytoplasmic PY motifs, and it is localized to the Golgi, endosomes, and multivesicular bodies (10). Ndfip1 also was shown to interact with Itch. Mice that are deficient in Ndfip1 develop inflammation in the skin and lungs similar to Itch−/− mice and die prematurely (11).

Nontypeable Haemophilus influenzae (NTHi) is a major cause of respiratory infections and is the most common colonizer of airways in patients with chronic obstructive pulmonary disease, leading to its exacerbation (12). NTHI was shown to induce an NF-κB–mediated inflammatory response through activation of the kinase Tak1 (13). In this article, we demonstrate that Ndfip1 regulates Itch ligase activity toward Tak1 by promoting the binding of the E2 UbcH7 and inhibits NTHI-induced airway inflammation.

Materials and Methods

Mice

Itch−/− and Ndfip1−/− mice were described previously (11, 14). C57BL/6 mice were purchased from Charles River Laboratory. All mice were housed in microisolator cages in the barrier facility of the Baylor Institute for Immunology Research. All experiments were performed in accordance with the guidelines of the Institutional Animal Care and Use Committee of Baylor Research Institute.

Plasmid construction and cell transfection

Flag-Itch, Flag-Tak1, hemagglutinin (HA)-Ub (wild-type [WT]), HX-Itch (CS30A), and Myc-Ndfip1 were described previously (14, 15). HA-Ube2L3 (UbcH7) was obtained from Addgene (cat. no. 27561). Myc–Ndfip1-ΔN, Myc–Ndfip1-ΔM, and Myc–Ndfip1-ΔC were created from Myc-Ndfip1 by

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subcloning into pcDNA3.1/myc-His (-) A (Invitrogen) between the Xhol and HindIII restriction sites. Flag–Ndfip1-D1–41 and Flag–Ndfip1-DPY were created from Myc–Ndfip1 by subcloning into pCMVTag2B between HindIII and Xhol sites. All clone sequences were confirmed. Transient transfection of 293T cells was performed using Lipofectamine 2000 (Invitrogen), according to the manufacturer’s instructions (14).

Protein identification by liquid chromatography-tandem mass spectrometry

Immunoprecipitated proteins were separated by SDS-PAGE. In-gel digestion with trypsin, followed by protein identification using liquid chromatography-tandem mass spectrometry, was performed as described elsewhere (16). Briefly, tryptic peptides were resolved on a nano-LC column (Magic AQ C18; Michrom Bioreources) and introduced into an Orbitrap mass spectrometer (Thermo Scientific). The Orbitrap was set to collect a high-resolution MS1 (FWHM 30,000@400 m/z), followed by data-dependent collision-induced dissociation spectra on the “top 9” ions in the linear ion trap. Spectra were searched against a human protein database (UniProt release 2011_05) using the X!Tandem/TPP software suite (17). Proteins identified with a ProteinProphet probability ≥ 0.9 false discovery rate < 2% were considered for further analysis.

Abs and reagents

The following Abs were used in this study: anti-c-Myc (sc-40; Santa Cruz), anti-Flag (F1804, F7425; Sigma), anti-Itch (611198; BD), anti-β-actin (A5441; Sigma), anti-Ub (sc-8017; Santa Cruz), anti-Xpress (R910-25; Invitrogen), anti- HA (sc-805; Santa Cruz), anti-UBE2G2 (sc-100613; Santa Cruz), anti-V5 Ab (R900-25; Invitrogen), anti-Tak1 (Sc-7976; Santa Cruz), and Lys48-specific anti-Ub (05-1307; Millipore). The Tak1 inhibitor [(5Z)-7-Oxozeaenol, 3064] (R960-25; Invitrogen), anti-Tak1 (Sc-7976; Santa Cruz), and Lys48-specific anti-Ub (05-1307; Millipore). The Tak1 inhibitor [(5Z)-7-Oxozaeanol, 3064] was purchased from Tocris Bioscience. Ready-SET-Go! ELISA kits for mouse IL-6, TNF, and IL-1β were purchased from eBioscience. TLR2/TLR4 inhibitor (OxPAPC, tlrl-oxpl) was purchased from Invitrogen.

Ubiquitination assay

293T cells were transfected with Flag-Tak1 and various constructs, as indicated. MG132 was added 8 h before cell lysis. Cells were washed three times with PBS and lysed in Nonidet P-40 lysis buffer. Immunoprecipitation was performed using anti-Flag Ab. Tak1-associated Ub was analyzed by immunoblot using Ab against HA, as we described previously (14).

Lentiviral transduction

Lentivirus transfection was performed as described earlier (14). Expression clones were created by Gateway cloning technology, according to the manufacturer’s instructions (Invitrogen). The full-length Ndfip1 expression clones were created first by PCR subcloning of Ndfip1 into the pENTR-3C entry vector (Invitrogen) between EcoRI and XhoI, followed by recombination by Gateway LR cloning into pLent6.2/N-Lumio/V5-DEST (Invitrogen). Expression clones for Ndfip1-D1–41 and Ndfip1-DPY deletion mutants were similarly prepared first by PCR subcloning into pENTR-3C entry vector (Invitrogen), followed by recombination by LR into pLent6.2/N-Lumio/V5-DEST (Invitrogen). The ViraPower lentiviral expression system (Invitrogen) was used for lentiviral transduction of bone marrow–derived macrophages (BMDMs). First, 293FT cells were used for packaging and production of viruses. BMDMs were incubated with the virus-containing supernatants overnight at 37°C in a 5% CO2 incubator.

NTHi lysate preparation and exposure

NTHi lysates were prepared as described earlier (18). In brief, bacteria were grown on chocolate agar plates at 37°C in the presence of 5% CO2 and inoculated in brain heart infusion broth supplemented with 3.5 μg/ml NAD and hemin. The bacterial lysate was prepared by sonication. The final protein concentration was adjusted to 2.5 mg/ml in PBS, and aliquots were frozen at −80°C. Mouse inhalation of NTHi lysate was performed with an Aeromist CA-209 nebulizer (CIS-US, Bedford, MA). In brief, a thawed NTHi lysate was placed in the nebulizer driven by 10 l/min of room air supplemented with 5% CO2 for 20 min.

Lung inflammation was scored by examining H&E-stained lung sections using a previously described five-point lung injury scoring system (peri-vascular and peribronchial inflammation, hyaline membranes, alveolar and interstitial infiltrates, and alveolar hemorrhage) (19).

Real-time PCR analysis

Total RNA was prepared using an RNasea Mini Kit (QIAGEN), followed by cDNA synthesis using a Verso cDNA Kit (Thermo Scientific). Quantitative real-time PCR was performed on a Mastercycler ep realplex (Eppendorf). LightCycler 480 SYBR Green I Master reaction mix (Roche) was used in a 20-μl reaction volume. The expression of individual genes was normalized to the expression of actin. Cycling conditions were 95°C for 2 min, followed by 50 cycles of 95°C for 15 s, 55°C for 15 s, and 72°C for 20 s.

![FIGURE 1. NTHi induces severe lung inflammation in Itch−/− and Ndfip1−/− mice. (A) H&E staining of WT, Itch−/−, and Ndfip1−/− mice lungs with and without multiple exposures to NTHi lysate. Scale bar, 25 μm. (B) Inflammation score. (C) Relative mRNA levels of proinflammatory cytokines IL-6, IL-1β, and TNF from the lung tissue of WT, Itch−/−, and Ndfip1−/− mice. Data are representative of three or more independent experiments.](http://www.jimmunol.org/)

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Statistical analysis

Data were analyzed with GraphPad Prism 4 software to determine statistical significance using one-way ANOVA. Data are expressed as mean ± SD. A p value < 0.05 was considered significant.

Results

Itch<sup>−/−</sup> and Ndfip1<sup>−/−</sup> mice are hyperresponsive to NTHi-induced lung inflammation

It was demonstrated that NTHi activated the Tak1–NF-κB pathway and initiated an inflammatory response in the lung (13). Tak1 activation was shown to be regulated by ubiquitination of lysine residues within Tak1 (20–24). Because we reported earlier that Itch targets Tak1 for ubiquitination (14), we sought to get molecular insights into Tak1 ubiquitination by a proteomics approach using mass spectrometry. We transiently transfected 293T cells with Myc-Itch, HA-Ub, and Flag-Tak1 and immunoprecipitated Tak1 using anti-Flag Ab. The immunoprecipitates were subjected to mass spectrometry analysis following in-gel trypsin digestion, and Ndfip1 was identified in the Tak1 complex (Supplemental Fig. 1).

Because Ndfip1 was shown to interact with Itch (11), and deficiency of Ndfip1 resulted in chronic inflammation (11), we hypothesized that Ndfip1 may cooperate with Itch to limit lung inflammation. To test this hypothesis, we adopted a mouse model of NTHi-induced airway inflammation. WT, Itch<sup>−/−</sup>, and Ndfip1<sup>−/−</sup> mice were exposed to aerosolized UV-treated NTHi lysate weekly.

**FIGURE 2.** Elevated proinflammatory cytokine production by Itch<sup>−/−</sup> and Ndfip1<sup>−/−</sup> BMDMs in response to NTHi-P6. (A) ELISA of IL-6, IL-1β, and TNF from the BMDMs of WT, Itch<sup>−/−</sup>, and Ndfip1<sup>−/−</sup> mice treated with NTHi outer membrane protein P6 for 24 h. (B) Relative mRNA levels of IL-6, IL-1β, and TNF from WT, Itch<sup>−/−</sup>, and Ndfip1<sup>−/−</sup> BMDMs treated with P6 in the presence or absence of 5Z-7-Oxozeaenol (50 nM). Data represent three independent experiments. (C) ELISA of IL-6, IL-1β, and TNF from culture supernatants of WT, Itch<sup>−/−</sup>, and Ndfip1<sup>−/−</sup> BMDMs treated with P6 in the presence or absence of 5Z-7-Oxozeaenol (50 nM). Data represent three independent experiments. (D) ELISA of IL-6, IL-1β, and TNF from culture supernatants of WT, Itch<sup>−/−</sup>, and Ndfip1<sup>−/−</sup> BMDMs treated with P6 in the presence or absence of OxPAPC (30 μg/ml). Data represent three independent experiments.
for 4 wk, as previously described (18). Histological analysis of the lung tissue showed aggravated inflammation in \( \text{Itch}^{--} \) and \( \text{Ndfip1}^{--} \) mice compared with WT mice (Fig. 1A, IB). Next, we analyzed the expression of proinflammatory cytokines in the lung tissues by real-time PCR. As shown in Fig. 1C, increased levels of TNF, IL-6, and IL-1\( \beta \) were observed in \( \text{Itch}^{--} \) and \( \text{Ndfip1}^{--} \) lung tissues exposed to NTHi, corroborating the histopathological phenotype of enhanced pulmonary inflammation.

Because myeloid-derived proinflammatory cytokines play crucial roles in NTHi-induced lung inflammation and chronic obstructive pulmonary disease (18, 25–28), we used FACS to sort CD11b\(^+\) cells from the lung tissues of WT, \( \text{Itch}^{--} \), and \( \text{Ndfip1}^{--} \) mice. Expression of proinflammatory cytokines was analyzed by real-time PCR. As shown in Supplemental Fig. 2A, we found significantly higher expression levels of IL-6, IL-1\( \beta \), and TNF in \( \text{Itch}^{--} \) and \( \text{Ndfip1}^{--} \) cells than in WT cells. These results suggested that Itch and Ndfip1 negatively regulated the production of proinflammatory cytokines during NTHi infection.

**NTHi-P6 promotes Itch and Ndfip1 association**

The outer membrane lipoprotein P6 was shown to bind to TLR2 and activate NF-kB via Tak1 (13). Therefore, we investigated whether Itch and Ndfip1 regulated signaling pathways downstream of the P6-TLR2 pathway during NTHi infection. We incubated WT, \( \text{Itch}^{--} \), and \( \text{Ndfip1}^{--} \) BMDMs with purified P6 for 24 h. The culture supernatants were assayed for IL-6, TNF, and IL-1\( \beta \) by ELISA. As shown in Fig. 2A, both \( \text{Itch}^{--} \) and \( \text{Ndfip1}^{--} \) macrophages secreted elevated levels of proinflammatory cytokines compared with WT macrophages. To confirm that the elevated proinflammatory cytokine expression was through the P6-TLR2 pathway (13), we used a TLR2 inhibitor, OxPAPC (29, 30). We found that OxPAPC significantly inhibited P6-induced proinflammatory cytokines in \( \text{Itch}^{--} \) and \( \text{Ndfip1}^{--} \) cells (Fig. 2B). To investigate whether a defect in the regulation of Tak1 led to enhanced production of proinflammatory cytokines, we preincubated the BMDMs with (5Z)-7-Oxozeaenol, a Tak1 selective inhibitor (31). We found that (5Z)-7-Oxozeaenol treatment markedly inhibited proinflammatory cytokine expression in \( \text{Itch}^{--} \) and \( \text{Ndfip1}^{--} \) cells in response to P6 (Fig. 2C, 2D). Further, (5Z)-7-Oxozeaenol treatment inhibited proinflammatory cytokine expression by NTHi-infected macrophages (Supplemental Fig. 2B).

Next, we investigated whether Itch and Ndfip1 associate with Tak1. We transiently transfected 293T cells with Flag-Tak1, Flag-Itch, and Myc-Ndfip1 and immunoprecipitated the cell lysates with anti-Flag Ab. The immunoprecipitates were blotted with anti-Myc and then reprobed with anti-Flag Ab. As shown in Fig. 3A, we
Ndfip1 acts as an adaptor for UbcH7 and Itch

The function of Itch is believed to be regulated at the point of substrate recognition through the WW domains (32). Itch ligase activity was shown to be enhanced by JNK1-mediated phosphorylation (32, 33). Because Itch is a HECT-type E3 ligase, which requires the formation of a thioester covalent attachment of Ub to a highly conserved cysteine residue in its HECT domain by the E2 enzyme (5), we hypothesized that Ndfip1 may regulate Itch ligase activity by recruiting Ub-loaded E2. To test this hypothesis, we checked whether Ndfip1 interacts with UbcH7, the E2 that forms the thioester linkage for Itch (34–36). Interestingly, we found that UbcH7 coprecipitated with Ndfip1 in P6-stimulated BMDMs (Fig. 4A). To confirm the specificity of UbcH7 as an E2 that is recruited by Ndfip1, we performed communoprecipitation using another E2, UBE2G2. As shown in Fig. 4B, Ndfip1 failed to interact with UBE2G2.

To further confirm that Ndfip1 recruits UbcH7 to Itch and facilitates Tak1 ubiquitination, we stimulated WT and \( Ndfip1^{−/−} \) BMDMs with P6. The cell lysates were immunoprecipitated with anti-Itch Ab, and the membranes were immunoblotted with anti-Uch7 and anti-Ndfip1 Abs. UbcH7 immunoprecipitated with anti-Itch Ab. By comparison, we observed no UbcH7-specific bands when Itch, Ndfip1, and Tak1 were coexpressed. This suggests that Ndfip1 promotes Itch ligase activity and enhances K48-linked ubiquitination of Tak1. We also tested whether Ndfip1 promoted K63-linked ubiquitination by transfecting Itch in the presence of Ndfip1 and an Ub mutant in which all of the lysine residues except K63 were mutated (HA-UbK63). As shown in Fig. 4C, increased levels of polyubiquitinated Tak1 were observed when Itch, Ndfip1, and Tak1 were coexpressed. This suggests that Ndfip1 promotes Itch ligase activity and enhances K48-linked ubiquitination of Tak1. We also tested whether Ndfip1 promoted K63-linked ubiquitination by transfecting Itch in the presence of Ndfip1 and an Ub mutant in which all of the lysine residues except K63 were mutated (HA-UbK63). As shown in Fig. 4D, increased levels of polyubiquitinated Tak1 were observed when Itch, Ndfip1, and Tak1 were coexpressed. This suggests that Ndfip1 promotes Itch ligase activity and enhances K48-linked ubiquitination of Tak1.

Because it was demonstrated that Ndfip1 enhances Itch ligase activity and regulates JunB ubiquitination during Th2 cell differentiation (11, 15), we tested whether Ndfip1 also enhances Tak1 ubiquitination. We transfected 293T cells with different combinations of Flag-Tak1, Myc-Itch, Myc-Ndfip1, HA-UbK48 (in which all of the lysine residues except K48 were mutated), and HX-Itch-C830A (ligase mutant). We immunoprecipitated the cell lysates with anti-Flag Ab and blotted the membranes with anti-HA Ab. As shown in Fig. 3D, increased levels of polyubiquitinated Tak1 were observed when Itch, Ndfip1, and Tak1 were coexpressed. This suggests that Ndfip1 promotes Itch ligase activity and enhances K48-linked ubiquitination of Tak1. We also tested whether Ndfip1 promoted K63-linked ubiquitination by transfecting Itch in the presence of Ndfip1 and an Ub mutant in which all of the lysine residues except K63 were mutated (HA-UbK63). As shown in Supplemental Fig. 3A, we did not detect K63-linked ubiquitination of Tak1. Together, these data suggest that Ndfip1 promotes K48-linked, but not K63-linked, ubiquitination of Tak1.

FIGURE 4. Ndfip1 acts as an adaptor for UbcH7 and Itch. (A) Immunoassay of lysates from P6-treated WT BMDMs, followed by immunoprecipitation with anti-Ndfip1 Ab and mouse IgG and immunoblot analysis with anti-UbcH7 or anti-Ndfip1 Abs. (B) Immunoassay of lysates from P6-treated WT BMDMs, followed by immunoprecipitation with anti-Ndfip1 Ab and immunoblot analysis with anti-UbcH7, anti-UBE2G2, and anti-Ndfip1 Abs. (C) Immunoassay of lysates from P6-treated WT and \( Ndfip1^{−/−} \) BMDMs, followed by immunoprecipitation with anti-Itch Ab and immunoblot analysis with anti-Uch7, anti-UBE2G2, and anti-Ndfip1 Abs. (D) Lysates were prepared from WT and \( Ndfip1^{−/−} \) lungs post-NTHi exposure and immunoprecipitated with anti-Itch Ab, followed by immunoblot analysis with anti-UbcH7, anti-Ndfip1, or anti-Itch Ab.

Because it was demonstrated that Ndfip1 enhances Itch ligase activity and regulates JunB ubiquitination during Th2 cell differentiation (11, 15), we tested whether Ndfip1 also enhances Tak1 ubiquitination. We transfected 293T cells with different combinations of Flag-Tak1, Myc-Itch, Myc-Ndfip1, HA-UbK48 (in which all of the lysine residues except K48 were mutated), and HX-Itch-C830A (ligase mutant). We immunoprecipitated the cell lysates with anti-Flag Ab and blotted the membranes with anti-HA Ab. As shown in Fig. 3D, increased levels of polyubiquitinated Tak1 were observed when Itch, Ndfip1, and Tak1 were coexpressed. This suggests that Ndfip1 promotes Itch ligase activity and enhances K48-linked ubiquitination of Tak1. We also tested whether Ndfip1 promoted K63-linked ubiquitination by transfecting Itch in the presence of Ndfip1 and an Ub mutant in which all of the lysine residues except K63 were mutated (HA-UbK63). As shown in Supplemental Fig. 3A, we did not detect K63-linked ubiquitination of Tak1. Together, these data suggest that Ndfip1 promotes K48-linked, but not K63-linked, ubiquitination of Tak1.
Figure 5. N-terminal end of Ndfip1 interacts with UbcH7. (A) 293T cells were transfected with Myc-Ndfip1, Myc–Ndfip1–ΔN, Myc–Ndfip1–ΔM, Myc–Ndfip1–ΔC, and HA-UbcH7 plasmids. Cells lysates were immunoprecipitated with anti-Myc Ab and immunoblotted using anti-HA and anti-Myc Abs. (B) 293T cells were cotransfected with Myc-Ndfip1, Myc–Ndfip1–ΔN, and Flag-Itch plasmids. Cells lysates were immunoprecipitated with anti-Flag Ab and immunoblotted using anti-Myc Ab. (C) 293T cells were transfected with Myc-Ndfip1, Myc–Ndfip1–Δ1–41, Myc–Ndfip1–ΔPY, Flag-Itch, and HA-UbcH7 plasmids. Cell lysates were immunoprecipitated with anti-Myc Ab and immunoblotted using anti-HA, anti-Flag, and anti-Myc Abs. (D) Ndfip1–/– BMDMs were reconstituted with full-length Ndfip1, Ndfip1–Δ1–41, or Ndfip1–ΔPY via lentiviral transduction. Cells lysates were immunoprecipitated with anti-V5 Ab and immunoblotted using anti-Itch, anti-UbcH7, and anti-V5 Abs. (E) Ndfip1–/– BMDMs were reconstituted with full-length Ndfip1, Ndfip1–Δ1–41, or Ndfip1–ΔPY via lentiviral transduction. Cell lysates were immunoprecipitated with anti-Tak1 Ab and immunoblotted using Apu2 (anti-Ub Lys 48 specific) and anti-Tak1 Abs. (F) Relative mRNA levels of proinflammatory cytokines IL-6, IL-1β, and TNF from BMDMs isolated from Ndfip1–/– mice reconstituted with full-length Ndfip1, Ndfip1–Δ1–41, or Ndfip1–ΔPY via lentiviral transduction and stimulated with P6 for 4 h. (G) ELISA of proinflammatory cytokines IL-6, IL-1β, and TNF from the culture supernatants of BMDMs isolated from Ndfip1–/– mice reconstituted with full-length Ndfip1, Ndfip1–Δ1–41, or Ndfip1–ΔPY via lentiviral transduction and stimulated with P6 for 24 h. (H) Model depicting the adaptor function of Ndfip1 for Itch and UbcH7 during Tak1 ubiquitination. n.s., not significant.
mutants, rescued elevated proinflammatory cytokine expression by reconstitution of full-length Ndfip1, but not Ndfip1 deletion mutants, in the presence of full-length Ndfip1 but not the Ndfip1 deletion mutants lacking aa 1–41 (Ndfip1-Δ1–41) and lacking the PY motif (aa 42–76; Ndfip1-ΔPY). We transfected 293T cells with UbcH7 and Itch along with full-length Ndfip1, Ndfip1-Δ1–41, or Ndfip1-ΔPY. As shown in Fig. 5C, Itch and UbcH7 coprecipitated in the presence of full-length Ndfip1 but not the Ndfip1-Δ1–41 mutant. This suggested that UbcH7 binds to the first 41 N-terminal amino acids, whereas Itch binds to the PY motifs (Fig. 5C).

Reconstitution of full-length Ndfip1, but not Ndfip1 mutants, rescues elevated proinflammatory cytokine expression by Ndfip1-Δ1–41 cells

To further investigate the role of Ndfip1 as an adaptor for UbcH7 and Itch during NTHi-induced inflammatory responses, we reconstituted Ndfip1-Δ1–41/BMDMs with full-length Ndfip1, Ndfip1-Δ1–41, or Ndfip1-ΔPY via lentiviral transduction. We stimulated the cells with P6 and analyzed Itch, Ndfip1, and UbcH7 interactions by coimmunoprecipitation experiments. As shown in Fig. 5D, full-length Ndfip1 interacted with Itch and UbcH7 in P6-treated cells. Ndfip1-ΔPY interacted with UbcH7 but not with Itch. Conversely, Ndfip1-Δ1–41 interacted with Itch but not with UbcH7. We further tested the effect of reconstituting Ndfip1-Δ1–41/BMDMs on Tak1 ubiquitination. The transduced Ndfip1-Δ1–41 cells were stimulated with P6 protein, and the endogenous Tak1 was immunoprecipitated using anti-Tak1 Ab, followed by immunoblotting with Apu2 Ab. As shown in Fig. 5E, reconstitution of full-length Ndfip1, but not the deletion mutants, restored the defect in Tak1 ubiquitination in Ndfip1-Δ1–41 cells. We also analyzed the level of proinflammatory cytokine expression in Ndfip1-Δ1–41/BMDMs. As shown in Fig. 5F, 5G, and Supplemental Fig. 4B, reconstitution of Ndfip1-Δ1–41 cells with full-length Ndfip1, but not with Ndfip1-Δ1–41 or Ndfip1-ΔPY mutants, rescued elevated proinflammatory cytokine expression by Ndfip1-Δ1–41/BMDMs. These results suggest that Ndfip1 plays a crucial role as an adaptor molecule bridging Itch and UbcH7 to facilitate termination of inflammation signaling (Fig. 5H).

Discussion

By virtue of its target substrates, Itch plays a crucial role in inflammatory signaling pathways (32, 37), but how its function is controlled remains unclear. In this article, we demonstrate that Itch ligase activity is regulated by Ndfip1 by facilitating the recruitment of the E2 enzyme UbcH7. Itch or Ndfip1 deficiency led to defects in Itch-mediated Tak1 ubiquitination.

NTHi is associated with chronic upper and lower respiratory disease and is the dominant species isolated from the lower airways of children and adults with chronic respiratory symptoms (38, 39). The inflammatory response after NTHi infection is characterized by the upregulation of proinflammatory cytokines, such as TNF and IL-1β, which is mediated by MAPK and NF-κB through TLRs (40). It was demonstrated that the NTHi membrane lipoprotein P6 activates Tak1, the upstream kinase that is responsible for NF-κB activation (13, 41). We demonstrated earlier that Itch forms an Ubc-editing complex with the deubiquitinase Cyld. This complex sequentially cleaves the K63-linked Ub chains on Tak1 and catalyzes K48-linked ubiquitination to promote Tak1 degradation (14). In this article, we demonstrate that Ndfip1 promotes Itch-mediated K48-linked ubiquitination by acting as an adaptor for UbcH7 and Itch (Fig. 5G). It is not known whether P6 induces K63-linked ubiquitination to activate Tak1 during the early phase of infection. Such a phenomenon was reported in the case of Helicobacter pylori infection, in which TRAF6 catalyzes K63-linked ubiquitination of Tak1 (20). It is possible that the Itch-Cyld complex switches K63-linked ubiquitination to K48-linked ubiquitination on Tak1 to inhibit NTHi-induced airway inflammation, and Ndfip1 may facilitate Itch function by recruiting Ub-loaded UbcH7.

Itch function was proposed to be regulated at the point of substrate recognition through its WW domains and via intramolecular autoinhibition (32). The autoinhibitory interaction between the WW domains and the HECT domain could prevent E2 binding or inhibit the translocation reaction between E2 and E3 (42). Upon activation of the cells, JNK1-mediated phosphorylation was shown to cause a conformational change that results in the destabilization of the inhibitory intramolecular interactions and promotes Itch ligase activity (33). Additionally, Ndfip1 was shown to enhance the Itch ligase activity (11). Our results suggest that Ndfip1 does it by recruiting UbcH7, suggesting that there is additional regulation of Itch function at the level of E2 recruitment. Our results from reconstitution of Ndfip1-Δ1–41 cells with Ndfip1 truncation mutants suggest that interaction of Ndfip1 with Itch, as well as UbcH7, is essential for effective termination of the inflammatory response. However, it remains unclear whether the Itch–Ndfip1 interaction or the UbcH7–Ndfip1 interaction plays a predominant role.

Regulation of Itch ligase activity by Ndfip1 may not be restricted to the TLR2 pathway. Our results suggest that LPS also promoted an Ndfip1–UbcH7 interaction in BMDMs (Supplemental Fig. 4B). Because NTHi is a Gram-negative bacterium with LPS as a component of its cell wall, it is likely that NTHi and Ndfip1 also regulate Tak1 ubiquitination to downmodulate TLR4-induced Tak1 activation in NTHi-infected macrophages. Whether Ndfip1 regulates Tak1 in other signaling pathways remains unknown. Furthermore, it remains unclear whether the interaction among Itch, Ndfip1, UbcH7, and Tak1 is direct. Further detailed molecular and genetic analyses will be required to determine the exact mechanisms of interaction among these proteins. A better understanding of Ndfip1-mediated regulation of Itch ligase activity is essential for inhibiting or enhancing Itch function in inflammatory pathways.

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Disclosures

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