Regulation of IL-4 Receptor Signaling by STUB1 in Lung Inflammation

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Abstract

Rationale: IL-4R α , the common receptor component for IL-4 and IL-13, plays a critical role in IL-4- and IL-13-mediated signaling pathways that regulate airway inflammation and remodeling. However, the regulatory mechanisms underlying IL-4R α turnover and its signal termination remain elusive.

Objectives: To evaluate the role of STUB1 (STIP1 homology and U-Box containing protein 1) in regulating IL-4R signaling in airway inflammation.

Methods: The roles of STUB1 in IL-4R α degradation and its signaling were investigated by immunoblot, immunoprecipitation, and flow cytometry. The involvement of STUB1 in airway inflammation was determined *in vivo* by measuring lung inflammatory cells infiltration, mucus production, serum IgE levels, and alveolar macrophage M2 activation in STUB1^{-/-} mice. STUB1 expression was evaluated in airway epithelium of patients with asthma and lung tissues of subjects with chronic obstructive pulmonary disease.

Measurements and Main Results: STUB1 interacted with IL-4R α and targeted it for ubiquitination-mediated proteasomal degradation, terminating IL-4 or IL-13 signaling. STUB1 knockout cells showed increased levels of IL-4R α and sustained STAT6 activation, whereas STUB1 overexpression reduced IL-4R α levels. Mice deficient in STUB1 had spontaneous airway inflammation, alternative M2 activation of alveolar macrophage, and increased serum IgE. STUB1 levels were increased in airways of subjects with asthma or chronic obstructive pulmonary disease, suggesting that up-regulation of STUB1 might be an important feedback mechanism to dampen IL-4R signaling in airway inflammation.

Conclusions: Our study identified a previously uncharacterized role for STUB1 in regulating IL-4R signaling, which might provide a new strategy for attenuating airway inflammation.

Keywords: IL-4R signaling; STUB1; airway inflammation

At a Glance Commentary

Scientific Knowledge on the Subject: IL-4R signaling plays a critical role in the pathogenesis of airway inflammation and other diseases, including helminth infections, rheumatoid arthritis, diabetes, and cancer. The molecular mechanisms underlying IL-4R α turnover and its signaling termination remain unclear.

What This Study Adds to the Field: We report here that STUB1 controls IL-4R signaling in airway inflammation. STUB1 interacted with and regulated IL-4R α by mediating its degradation, thus influencing the IL-4R α -STAT6 signaling. STUB1 deficiency induced airway inflammation, IgE production, and alternative activation of macrophages in mice. STUB1 levels were increased in airway epithelium of patients with asthma and in lung tissues of subjects with chronic obstructive lung diseases, suggesting that up-regulation of STUB1 might be an important feedback mechanism to dampen IL-4R signaling in airway inflammation. Our study revealed a new role for STUB1 in regulating IL-4R signaling, which might provide a new strategy for attenuating airway inflammation.

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As a key determinant of Th2 immune response and inflammation, IL-4R signaling pathway plays a critical role in the pathogenesis of asthmatic airway inflammation (1, 2) and other diseases including helminth infections (3), rheumatoid arthritis (4), diabetes (5), and cancer (6, 7). IL-4R α , a common receptor subunit shared by IL-4 and IL-13, is essential for signal transduction by both cytokines. IL-4R α associates with cytokine receptor common γ chain (γ c) to form type I IL-4R receptor, a receptor exclusive for IL-4, or pairs with IL-13R α 1 to form type II IL-4 receptor, which is activated by both IL-4 and IL-13 (1). The binding of IL-4 or IL-13 to IL-4 receptors induces activation of Janus kinases, which then initiates several signaling cascades that result in the binding of phosphorylated signal transducer and activator of transcription 6 (STAT6) to the promoters of the IL-4 and IL-13 responsive genes. In concert, activation of Janus kinases also leads to phosphorylation of insulin receptor substrates, which activates the PI3K signaling pathway (1). In asthma, IL-4R α -coupled signaling pathways mediate airway inflammation, remodeling and hyperresponsiveness, mucus hypersecretion, increased IgE, and subepithelial fibrosis; all are key features of asthmatic pathophysiology (1, 2). Gene-gene interactions between IL-4R α and IL-4 or IL-13 have been shown to increase the risk of asthma (8, 9). Furthermore, signaling through IL-4 is required for Th2 cell stimulation of mucus production, because Th2 cells do not induce mucus production in IL-4R α -deficient mice (10). Finally, treatment of mice with IL-4R α neutralizing antibodies or soluble IL-4R α inhibited the development of asthma phenotype (11, 12). Given the critical role of IL-4R signaling in airway inflammation and in asthma pathogenesis, a better understanding of the regulatory mechanisms underlying IL-4R α turnover and its signal termination will be crucial.

STIP1 homology and U-Box containing protein 1 (STUB1), also known as CHIP (C terminus of HSC70-interacting protein), is a chaperone-dependent E3 ubiquitin ligase (13). STUB1 interacts with Hsp70 and Hsp90 chaperones through its N-terminal tetratricopeptide repeat (TPR) domain and ubiquitinates client proteins with the C-terminal U-box (13). The function of CHIP is highly dependent on its capability to bind to Hsp70 and Hsp90.

A chaperone-binding domain mutant, STUB1-K30A, fails to interact with client proteins, implying that the interaction between STUB1 and its substrates is indirect and mediated by chaperone intermediate. Some of the client proteins include mutants of cystic fibrosis transconductance regulator, polyglutamine-expanded huntingtin, ataxin, and inducible nitric oxide synthase (14–17). In particular, STUB1 has been shown to mediate the ubiquitination and proteasomal degradation of receptors, such as Toll-like receptor 4, estrogen receptor α , mineralocorticoid receptor, Met receptor, and growth hormone receptor (18–22).

A recent genetic study has linked STUB1 to IL-4 and IL-4R α genes as part of an interconnected and interactive gene network that influences childhood asthma (23). Ingenuity pathway analysis demonstrated that the above interacting genes are involved in related biologic activities and functional commonalities. In this study, we showed that STUB1 interacted with and promoted ubiquitin-dependent degradation of IL-4R α . STUB1 deficiency resulted in sustained STAT6 activation and thereby induced airway inflammation, IgE production, and alternative activation of alveolar macrophage. Thus, STUB1 is an important regulator of lung inflammation.

Some of the results of these studies have been previously reported in the form of abstracts (24, 25).

Methods

Mice

Wild-type and *STUB1*^{-/-} mice were obtained from Texas A&M Institute for Genomic Medicine (college Station, TX). The genetic background of the mice is a cross of the 129S5/SvEvBrd and C57BL/6 strains. *STUB1* knockout mice were generated by crossing male *STUB1* heterozygous (*STUB1*^{+/-}) mouse with female *STUB1* heterozygous (*STUB1*^{+/-}) mouse. Genotypically homozygous normal siblings (*STUB1*^{+/+}) of *STUB1* knockout mice served as control animals for this study. Mice were maintained in a pathogen-free animal facility at Baylor College of Medicine. All experimental protocols were approved by the Institutional Animal Care and Use Committee of Baylor College of Medicine and followed National Institutes of Health animal use guidelines.

Cell Culture

Mouse embryo fibroblasts (MEFs) were generated from Day 13.5 embryos according to standard protocols and maintained in improved modified Eagle medium supplemented with 10% fetal bovine serum. Mouse primary tracheal epithelial cells were isolated as previously described (26) and described in the online supplement. A549 cells were obtained from American Type Culture Collection (Manassas, VA) and were grown in F12K medium supplemented with 10% fetal bovine serum. Normal human bronchial epithelial cells were obtained from Lonza (Houston, TX) and maintained in bronchial epithelial cell growth medium.

Plasmids and Transfections

Plasmid expressing full-length human IL-4R α cDNA was from Origene (Rockville, MD). Plasmid expressing human wild-type STUB1, STUB1 mutants H260Q and K30A, was kindly provided by Dr. Len Neckers. Recombinant lentivirus expressing STUB1 was generated by America Pharma Source (Gaithersburg, MD). Transient transfection of plasmids was performed using Lipofectamine LTX and PLUS (Life Technologies, Carlsbad, CA) into 70–80% confluent cells.

Immunoblot Analysis

Proteins were extracted from cells or lung tissues using RIPA buffer. Extracted proteins were separated, electroblotted, and incubated with primary antibodies. Immunoreactive bands were visualized and quantified by fluorescence emission using the Odyssey IR Imaging System (LI-COR Biosciences, Lincoln, NE). Antibodies used are listed in the online supplement.

Immunoprecipitation

Cells were lysed on ice (20 mM Tris-HCl, pH 8.0, 100 mM NaCl, 1 mM ethylenediaminetetraacetic acid, 0.5% NP-40, and protease inhibitor cocktail), clarified, and incubated with primary antibodies overnight at 4°C. Immunoprecipitates were harvested by Dynabeads protein G (Life Technologies) and beads were washed before elution with Lamml buffer. For experiments to examine ubiquitination of IL-4R α , 5 mM NEM was added to minimize deubiquitination of IL-4R α during cell lysis. Antibodies used are listed in the online supplement.

Pulse-Chase Analysis

A549 cells cotransfected with a plasmid encoding Flag-IL-4R α and either a control vector or a construct encoding His-STUB1 were pulsed with 0.25 mCi/ml 35 S-labeled methionine-cysteine mixture for 1 hour and terminated by Dulbecco's modified Eagle's medium with excess unlabeled methionine and cysteine (300 mg/L) before harvested at various time points. Cell lysates were immunoprecipitated with Flag antibody (Sigma, St. Louis, MO). Eluted proteins were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Gel bands representing 35 S-labeled IL-4R α were quantitated using a phosphorimager.

Bronchoalveolar Lavage Analysis

The mouse trachea was cannulated and lungs were lavaged three times with 0.8 ml of cold phosphate-buffered saline. The recovered fluid aliquots were pooled and cells were counted. Cytospin preparations were stained with Hema-3 reagents. At least 200 cells per slide were examined microscopically and characterized as neutrophils, lymphocytes, macrophages, or eosinophils.

Flow Cytometry

IL-4R α expression on cell surface of MEFs was determined by Fc blocking and then staining with PE-anti-IL-4R α antibody (BD

Biosciences, San Jose, CA). In the experiment of detecting IL-4R α cell surface expression in IL-4- and IL-13-treated MEFs, cells were washed with ice-cold acid stripping buffer (50 mM glycine, 150 mM NaCl, pH 2.5) to remove cell surface IL-4 and IL-13 before IL-4R α staining. Bronchoalveolar lavage (BAL) macrophages activation markers were determined by Fc blocking and then staining with APC-Cy7-anti-F4/80 (eBioscience, San Diego, CA), and fluorescein isothiocyanate-anti-CD206 (Biolegend, San Diego, CA) or APC-anti-Arginase 1 (R&D Systems, Minneapolis, MN) antibodies. Flow cytometry analysis was performed with LSRFortessa (BD

Figure 1. STIP1 homology and U-Box-containing protein 1 (STUB1) down-regulates IL-4R α levels. Expression of IL-4R α was analyzed by immunoblot (A and C) or flow cytometry (B) in mouse embryonic fibroblasts (MEF), lung tissues (six mice per group), or tracheal epithelial cells from wild-type (WT), STUB1^{+/-}, or STUB1^{-/-} mice. (D) Immunoblot analysis of IL-4R α in MEF infected with a recombinant lentivirus expressing STUB1 or a control lentivirus for 48 hours or in A549 cells cotransfected with plasmid encoding IL-4R α together with either a control vector or plasmid encoding His-STUB1 for 48 hours. (E) A549 cells cotransfected with plasmids encoding Flag-IL-4R α and STUB1 were pulsed with [35 S]methionine-cysteine and chased with unlabeled medium at indicated time points. IL-4R α was immunoprecipitated with α -Flag antibody, eluted, and analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Bands representing 35 S-labeled IL-4R α were quantified. (F) WT and STUB1^{-/-} MEF were treated with 25 μ M cycloheximide for indicated periods and cell lysates were subjected to immunoblot to evaluate IL-4R α . **Nonspecific band. Graph data are mean $\pm$ SD, * P < 0.05, ** P < 0.01. Representative blots are shown from at least three independent experiments. GAPDH = glyceraldehyde phosphate dehydrogenase.

Biosciences), and the data were analyzed with FlowJo software (Three Star Inc).

ELISA

Serum IgE levels were detected by BD OptEIA Mouse IgE ELISA kit (BD Biosciences) according to the manufacturer's protocol.

Histology

Mice lungs were inflated with 10% paraformaldehyde and fixed overnight. Tissues were subsequently embedded in paraffin, sectioned, and stained with hematoxylin and eosin or periodic acid Schiff for analysis. The evaluation of peribronchiolar inflammation and mucus production is described in the online supplement.

Tissue Immunohistochemistry and Immunofluorescence

Immunohistochemistry and immunofluorescence staining of mice or human lung tissues are described in the online supplement.

Quantitative Real-Time Polymerase Chain Reaction

Total RNA was isolated using the RNeasy kit (Qiagen, Germantown, MD) and reverse transcribed to cDNA using SuperScript III reverse transcriptase (Life Technologies). Quantitative polymerase chain reaction was performed with specific primer sets. Results were normalized to the expression levels of

glyceraldehyde phosphate dehydrogenase or β -actin and relative expression was calculated by the $2^{-\Delta\Delta CT}$ method. Primers are listed in the online supplement.

Human Subjects

Studies on children with asthma, adults with asthma, and patients with chronic obstructive pulmonary disease (COPD) were approved by the Institutional Review Board of University of Cincinnati or Baylor College of Medicine. Details of patient recruitment, sample collection, and processing are described in the online supplement.

Statistics

Statistical analysis for pulse-chase and cycloheximide-treatment experiments was performed using two-way analysis of variance. Results of other experiments were analyzed by unpaired Student *t* test. Statistical significance is displayed as **P* less than 0.05 or ***P* less than 0.01. All blots and data are representative of at least three independent experiments.

Results

STUB1 Down-regulates IL-4R α Levels

A recent study revealed a genetic linkage between the predisposition of childhood asthma and a gene network that interconnects IL-4, IL-4R α , and STUB1

(23). Because STUB1 possesses E3 ubiquitin ligase activity, we first hypothesized that STUB1 might be involved in the degradation of IL-4R α and hence regulates IL-4- and IL-13-mediated signaling through the IL-4R α . To test this hypothesis, we evaluated IL-4R α expression in MEF from STUB1 knockout (*STUB1*^{-/-}) mice and their wild-type counterparts. IL-4R α protein levels were significantly higher in *STUB1*^{-/-} MEF, compared with wild-type MEF (Figure 1A), whereas there was no significant difference in IL-4R α mRNA levels (see Figure E1A in the online supplement). These data suggested that IL-4R α might be up-regulated in *STUB1*^{-/-} MEF through post-transcriptional mechanisms. In contrast, levels of the common γ chain (γ c) were not changed in bone marrow-derived macrophages or MEFs from *STUB1*^{-/-} mice (Figure E1B). *STUB1*^{-/-} and wild-type MEF also had similar levels of IL-13R α 1 (Figure E1C). Thus, our data suggested that in IL-4R signaling pathway, STUB1 specifically targets the degradation of IL-4R α but not IL-13R α 1 and γ c. Flow cytometry analysis confirmed that cell surface expression of IL-4R α was about threefold higher in *STUB1*^{-/-} MEF than that in wild-type MEF (Figure 1B). Increased IL-4R α was also observed in lung tissues, tracheal epithelial cells, and tracheal fibroblasts of STUB1-deficient mice (Figures 1C and E1D). Conversely, ectopic expression of STUB1 decreased IL-4R α in MEF or A549

Figure 2. STUB1 homology and U-Box containing protein 1 (STUB1) interacts with IL-4R α . (A) Cell lysates of wild-type (WT) mouse embryonic fibroblasts cells were immunoprecipitated (IP) with antibodies against STUB1 (top) or IL-4R α (bottom) and aliquots of IP were analyzed by immunoblot. Immunoprecipitation with IgG antibody was used as a negative control. (B) Schematic diagram of WT STUB1 and mutant constructs. WT STUB1 contains two functional domains: an N-terminal tetratricopeptide repeat (TPR) domain interacting with chaperones and a C-terminal U-box domain with E3 ubiquitin ligase activity. In mutant K30A, the lysine residue at position 30 within the TPR domain was substituted with alanine. In mutant H260Q, the histidine residue at position 260 within the U-box domain was substituted with glutamine. (C) A549 cells were cotransfected with plasmid encoding Flag-IL-4R α and with either an empty vector or a construct encoding His-STUB1, His-STUB1-H260Q (HQ), or His-STUB1-K30A (KA). Twenty-four hours after transfection, cells were lysed and subjected to IP with antibodies against IgG, Flag, or His. Aliquots of IP proteins were analyzed by immunoblot. Representative blots are shown from at least three independent experiments.

cells, a human lung adenocarcinoma epithelial cell line (Figure 1D). We then investigated the effects of STUB1 on the stability of IL-4R α . Overexpression of STUB1 caused a faster degradation of IL-4R α (Figure 1E), whereas knockout of STUB1 stabilized IL-4R α (Figure 1F). Taken together, these results suggest that STUB1 down-regulates IL-4R α by accelerating its degradation.

STUB1 Interacts with IL-4R α

STUB1 mediates substrate ubiquitination and degradation by the proteasome through its interaction with molecular chaperones (13). We examined if STUB1 interacts with IL-4R α . We found that STUB1 colocalized with IL-4R α in human lung bronchioles (Figure E2). Coimmunoprecipitation assay, using STUB1 antibody to capture protein

complexes in MEF lysates, showed that IL-4R α coprecipitated with STUB1 (Figure 2A). In reciprocal experiments, antibody against IL-4R α coprecipitated STUB1. These data indicate that endogenous STUB1 interacts with IL-4R α *in vivo*. To further characterize IL-4R α –STUB1 interaction, experiments were done using constructs encoding IL-4R α , wild-type STUB1, and two functional mutants of STUB1 (Figure 2B). STUB1-K30A has a Lys-to-Ala substitution in the TPR chaperone-binding domain and therefore fails to bind to chaperones. STUB1-H260Q has a His-to-Gln substitution in the U-box domain of STUB1 and therefore loses its E3 ubiquitin ligase activity (27). A549 cells were cotransfected for 24 hours with Flag-tagged IL-4R α and a construct encoding either

His-tagged wild-type STUB1 (His-STUB1), His-STUB1-H260Q, or His-STUB1-K30A. As shown in Figure 2C, when IL-4R α was immunoprecipitated, using FLAG antibody, wild-type STUB1 and STUB1-H260Q but not STUB1-K30A were coprecipitated. Similarly, when wild-type STUB1 or STUB1-H260Q but not STUB1-K30A was immunoprecipitated by His antibody, IL-4R α was coprecipitated. These data suggested that STUB1-K30A failed to interact with IL-4R α . Because STUB1-K30A failed to bind to Hsp70, our results indicate that the chaperone binding activity of STUB1 is critical for its interaction with IL-4R α . STUB1-H260Q mutant exhibited a stronger interaction with IL-4R α than wild-type STUB1, as previously described for other STUB1 substrates (17), suggesting that the interaction between STUB1 and

Figure 3. STUB1 homology and U-Box containing protein 1 (STUB1) promotes IL-4R α proteasomal degradation by the ubiquitin–proteasome pathway. (A) Immunoblot analysis of Flag-IL-4R α and His-STUB1 proteins in A549 cells cotransfected for 24 hours with plasmid encoding Flag-IL-4R α and plasmid encoding His-STUB1, His-STUB1-H260Q (HQ), or His-STUB1-K30A (KA). (B) Immunoblot analysis of IL-4R α in mouse embryonic fibroblasts treated with MG132 (50 μ M) or lactacystin (Lac, 10 μ M) for 2 hours. (C) A549 cells were cotransfected, as described in A. Cells were then incubated with 10 μ M MG132 for 3 hours and cell lysates were subjected to immunoprecipitation (IP) with α -Flag antibody. Immunoprecipitates were probed with α -ubiquitin (α -Ub) antibody. *Nonspecific band. Bottom of each figure: Quantified analysis of Western blots with IL-4R α expression normalized to glyceraldehyde phosphate dehydrogenase (GAPDH) or β -actin. Data are mean $\pm$ SD; n = 3; * P < 0.05, ** P < 0.01. Representative blots are shown from at least three independent experiments. WT, wild-type.

Figure 4. (See following page for figure legend.)

IL-4R α either becomes stronger or lasts longer in the absence of ubiquitin ligase activity of STUB1. Interestingly, the binding of IL-4R α to Hsp70 was not affected by the STUB1 mutations either in the TPR chaperone-binding domain or in the U-box domain, suggesting that IL-4R α might directly interact with Hsp70 without the involvement of STUB1. Overall, these results indicate that STUB1 is associated with IL-4R α in cells and the association requires a chaperone intermediate.

STUB1 Promotes IL-4R α Degradation via the Ubiquitin-Proteasome Pathway

We hypothesized that STUB1 facilitates the chaperone-dependent targeting of IL-4R α to the ubiquitin-proteasome degradation pathway. Consistent with this hypothesis, overexpression of wild-type STUB1 significantly reduced levels of IL-4R α in A549 or HeLa cells, whereas H260Q and K30A mutants failed to down-regulate IL-4R α (Figures 3A and E3). STUB1-H260Q functions as a dominant-negative E3 ligase and STUB1-K30A does not bind Hsp90 or Hsp70 and fails to interact with IL-4R α . These data suggest that both the chaperone binding and ubiquitin ligase functions of STUB1 are required for its effect on IL-4R α . We next examined if IL-4R α degradation is mediated by the proteasome pathway. Inhibition of the proteasome by either MG132 or lactacystin led to accumulation of IL-4R α in wild-type but not in *STUB1*^{-/-} MEF (Figure 3B), suggesting that IL-4R α undergoes proteasomal degradation mediated by STUB1. Because ubiquitination is a sorting signal for targeting the substrate to the proteasome, we investigated if IL-4R α is ubiquitinated by STUB1. Overexpression of wild-type STUB1 promoted IL-4R α ubiquitination, whereas neither H260Q nor K30A mutant significantly affected levels of ubiquitinated IL-4R α (Figure 3C). These results indicate that STUB1 targets IL-4R α

for degradation through the ubiquitin proteasome pathway.

STUB1 Mediates IL-4- or IL-13-induced IL-4R α Degradation

Recent studies have revealed that ligand-induced internalization and endocytosis of cytokine receptors are mediated by the ubiquitin proteasome pathway (28, 29). We therefore sought to determine whether or not STUB1 is involved in ligand-induced degradation of IL-4R α . Treatment of MEF with IL-4 or IL-13 caused reduction of IL-4R α on the cell surface and in cell lysates in a dose- and time-dependent manner (Figures 4A–4C). Pretreatment with the proteasome inhibitor MG-132 blocked IL-4- or IL-13-induced IL-4R α destabilization (Figure 4C), suggesting that the process is mediated by the proteasome pathway. Importantly, IL-4 or IL-13 treatment failed to reduce IL-4R α expression in *STUB1*^{-/-} MEF (Figure 4D), suggesting that STUB1 is required for cytokine-induced IL-4R α degradation. We further found that STUB1 was also required for IL-4- or IL-13-induced polyubiquitination of IL-4R α in MEF (Figure 4E). These results suggest that IL-4- or IL-13-induced degradation of IL-4R α is mediated by STUB1. Such mechanism might serve as a regulatory feedback to regulate steady-state level of the IL-4R α and to terminate cytokine signaling.

Sustained STAT6 Activation in STUB1 Knockout Cells

STAT6 is one of the major downstream signaling molecules of IL-4R α . Having established the role of STUB1 in IL-4R α degradation, we attempted to determine if STUB1 regulates activation of STAT6. We examined the effect of IL-4 on STAT6 activation, as evidenced by Y641 phosphorylation, in *STUB1*^{-/-} and wild-type cells. As shown in Figure 5A, IL-4 treatment induced STAT6 phosphorylation in both wild-type and *STUB1*^{-/-} MEF. By

45 minutes post-stimulation, STAT6 phosphorylation was markedly decayed in wild-type MEF. In contrast, there was no such signal decay in *STUB1*^{-/-} MEF, indicating that STUB1 is required for termination of STAT6 activation. To confirm these findings, we treated MEF, differentiated tracheal epithelial cells, and bone marrow-derived macrophages with IL-4 or IL-13 for 30 minutes, and then washed out the cytokines. After removal of cytokines, there was rapid decay in phosphorylated STAT6 in wild-type cells. In contrast, decay of phosphorylated STAT6 was much slower in *STUB1*^{-/-} cells (Figures 5B–5D). These results demonstrate that STUB1 is required for termination of STAT6 activation, after induction by IL-4 or IL-13.

Increased Airway Inflammation and IgE Production in STUB1 Knockout Mice

Our data suggested that STUB1 knockout cells had sustained IL-4R α –STAT6 signaling in response to cytokine stimulation. We then investigated if STUB1 knockout mice have evidence of lung inflammation or increased serum IgE levels; both are known consequences of STAT6 activation (2). In the absence of immunologic sensitization and challenge, *STUB1*^{-/-} mice showed an increase in inflammatory cells in bronchioles and alveolar space, compared with wild-type mice (Figures 6A and 6B). The predominate cell types in both the peribronchial and alveolar inflammatory infiltrates are macrophages. In addition to inflammatory cells, we found a significant increase in periodic acid Schiff-positive cells and elevated levels of MUC5AC in bronchi of *STUB1*^{-/-} mice (Figures 6C and 6D). Finally, serum IgE levels were significantly increased in *STUB1*^{-/-} mice (Figure 6E). Thus, coinciding with increased STAT6 signaling in these mice, STUB1 knockout mice exhibit increased

Figure 4. STIP1 homology and U-Box containing protein 1 (STUB1) mediates IL-4- and IL-13-induced degradation of IL-4R α by the ubiquitin-proteasome pathway. (A) Flow cytometry analysis of IL-4R α expression on the surface of mouse embryonic fibroblasts (MEF) treated for 3 hours with IL-4 or IL-13 (0–50 ng/ml). Representative plots and quantitative graphs are shown. Data are mean $\pm$ SD; $n = 3$; $*P < 0.05$. (B) Immunoblot analysis of IL-4R α expression in MEF treated with 0–50 ng/ml IL-4 or IL-13 for 3 hours. (C) Immunoblot analysis of IL-4R α expression in MEF, which were pretreated with or without 10 μ M of MG132 and then treated with 50 ng/ml IL-4 or IL-13 for 0–3 hours. (D) Immunoblot analysis of IL-4R α expression in wild-type (WT) and *STUB1*^{-/-} MEF treated with 50 ng/ml IL-4 or IL-13 for 0–3 hours. (E) WT and *STUB1*^{-/-} MEF were treated with 50 ng/ml IL-4 or IL-13 for 3 hours. Cell lysates were immunoprecipitated with α -IL-4R α antibody and the immunoprecipitates were probed with α -ubiquitin (α -Ub) antibody. *Nonspecific band. Bottom of B–D: Quantified analysis of Western blots with IL-4R α expression normalized to glyceraldehyde phosphate dehydrogenase (GAPDH). Data are mean $\pm$ SD; $n = 3$; $*P < 0.05$, $**P < 0.01$. Representative blots are shown from at least three independent experiments.

Figure 5. STIP1 homology and U-Box containing protein 1 (STUB1) deficiency sustains signal transducer and activator of transcription 6 (STAT6) activation. (A) Mouse embryonic fibroblasts (MEF) were serum starved for 1 hour and then treated with 50 ng/ml of IL-4 for various time periods. (B–D) MEF, differentiated tracheal epithelial cells, or bone marrow derived macrophages (BMDMs) were serum starved for 1 hour, treated with 50 ng/ml of IL-4 or IL-13 for 30 minutes, washed with phosphate-buffered saline, and then incubated in serum-free media for 30 or 60 minutes. Cells were lysed and immunoblot analysis was performed to detect phosphorylated (p)-STAT6 and STAT6. **Nonspecific band. Right panel of each figure: Quantified analysis of Western blots with p-STAT6 expression normalized to glyceraldehyde phosphate dehydrogenase (GAPDH). Data are mean $\pm$ SD; $n = 3$; * $P < 0.05$; ** $P < 0.01$. Representative blots are shown from at least three independent experiments. WT, wild-type.

airway inflammation and IgE production. To further determine whether increased mucus, IgE, and inflammation in *STUB1*^{-/-} mice is caused by altered expression of IL-4R α , *STUB1*^{-/-} mice were intraperitoneally administered with anti-IL-4R α mAb, a specific mouse IL-4R α neutralizing antibody, or isotype IgG (as

control) twice within a week. We found that anti-IL-4R α mAb significantly reduced IgE levels, inflammatory infiltrates, and mucus production in *STUB1*^{-/-} mice (Figure E4), suggesting that the increased IgE, mucus production, and airway inflammation in *STUB1*^{-/-} mice was at least partially caused by the dysregulation of IL-4R α .

STUB1 Deficiency Is Associated with Alternative Activation of Macrophage

Next we evaluated total and differential cell count in BAL in wild-type and *STUB1*^{-/-} mice. Both total cell count and percentage of macrophages were significantly higher in BAL from *STUB1*^{-/-} mice compared with wild-type mice (Figures 7A and 7B).

Figure 6. Lung inflammation, mucus overproduction, and increased serum IgE levels in STUB1 homology and U-Box containing protein 1 (STUB1) knockout mice. (A) Lung sections from wild-type (WT) and STUB1^{-/-} mice were stained with hematoxylin and eosin. Arrows indicate representative infiltration of inflammatory cells. The right images are the amplification of the black rectangle. (B) Inflammation was scored as described in the online supplement. (C) Lung sections were stained with periodic acid Schiff (PAS) or MUC5AC antibody. Arrows indicate representative cells with positive staining. (D) Quantitative analyses are shown. (E) IgE in mouse serum was measured by ELISA. Data are mean $\pm$ SD; $n = 4$ –6 mice per group; $**P < 0.01$; scale bar, 20 μ M.

Importantly, alveolar macrophages from STUB1^{-/-} mice exhibited a highly vacuolated, foamy, and granular morphology, which was not observed in those from wild-type mice (Figure 7C). Such phenotype has been previously described to be associated with alternative activation of macrophages (30). To confirm this finding, we evaluated the expression of mannose receptor (CD206) and Arginase 1, the markers of alternative macrophage activation (31). Flow cytometry analysis showed that the percentages of F4/80⁺ macrophages expressing CD206 and Arginase 1 were higher in BAL from STUB1^{-/-} mice (Figures 7D and 7E). Percentage of F4/80⁺ macrophages

expressing MHC-II was lower in BAL from STUB1^{-/-} mice (Figure 7F). Taken together, these results suggest that STUB1 deficiency is associated with alternative activation of macrophage in mice.

STUB1 Is Up-regulated in the Airways of Subjects with Asthma or COPD

The above data suggested that STUB1 deficiency resulted in increased IL-4R signaling, sustained STAT6 activation, elevated serum IgE, increased airway inflammation, and alternative activation of macrophages, which are common features of airway inflammation. Given such phenotype in STUB1^{-/-} mice, and the recent finding of genetic linkage of STUB1

to childhood asthma (23), we reasoned that STUB1 levels might be altered in patients with asthma. We examined STUB1 mRNA level in nasal respiratory cells of stable children and adult with asthma and their age-matched control subjects. Notably, the children and adult cohorts of people with asthma and their control subjects were studied independently at the University of Cincinnati and Baylor College of Medicine, respectively. STUB1 mRNA levels were significantly increased in children and adult subjects with asthma (Figure 8A). IL-4R α expression was also up-regulated in adults with asthma, which is consistent with the previous observation that expression of

Figure 7. Alternative activation of alveolar macrophages in STUB1 homology and U-Box containing protein 1 (STUB1) knockout mice. Total (A) and differential (B) cell count of bronchoalveolar lavage (BAL) from wild-type (WT) and *STUB1*^{-/-} mice were determined. (C) Representative images of BAL cytopsin preparations are shown. Arrows indicate enlarged, foamy, and granular macrophages. (D–F) Double-positive populations of F4/80⁺/CD206⁺ (D), F4/80⁺/Arginase 1⁺ (E), F4/80⁺/MHC II⁺ (F) macrophages in BAL were quantified by flow cytometry. Data are mean $\pm$ SD; n = 3–5 mice per group; **P* < 0.05; ***P* < 0.01; scale bar, 20 μ M.

IL-4R α was enhanced in bronchial biopsy specimens from atopic and nonatopic subjects with asthma (32).

We then examined the expression of STUB1 and IL-4R α in subjects with COPD, another common chronic inflammatory airway disease. In COPD, the degree of airway inflammation relative to parenchymal lung tissue destruction may vary depending on the stage of the disease, with inflammation being more predominant in early stages to be replaced by lung destruction in later stages (33). Therefore, we evaluated lung tissues obtained from subjects with different disease severities, as evaluated by the FEV₁. We found that, in subjects with early stage of mild COPD (FEV₁ $\geq$ 80% predicted), both STUB1 and IL-4R α were significantly up-regulated in lung tissues (Figures 8B and Figure E5A). This result is consistent

with the previous observations in subjects with mild COPD that alveolar macrophages are partially M2 polarized (34), because the alternative M2 macrophages are activated by IL-4 and IL-13 through IL-4R α –STAT6 pathway. However, expression of STUB1 and IL-4R α did not change in subjects with COPD with moderate (FEV₁ 50–80% predicted) or later stage (FEV₁ < 50% predicted) of the disease (Figures E5B–E5D). The above data suggested that up-regulation of STUB1 is associated with increased levels of IL-4R α in lung inflammation. We hypothesized that STUB1 might be up-regulated in response to increased IL-4R signaling through a feedback mechanism. To test this hypothesis in a cellular model, we treated normal human bronchial epithelial cells and MEF with IFN- γ or LPS; both are inflammatory mediators

and IFN- γ has been shown to up-regulate IL-4R α expression in airway epithelium (35). Indeed, up-regulation of IL-4R α by IFN- γ or LPS increased STUB1 expression (Figure E6). Taken together, these results support the hypothesis that STUB1 might be up-regulated through a feedback mechanism to dampen the increased IL-4R α levels in inflammatory airway diseases.

Discussion

This study has several novel findings. First, it provides a novel pathway for regulation of IL-4R signaling by the ubiquitin ligase STUB1. Second, it reveals the underlying mechanism for such regulation. Third, it reveals that in *STUB1*^{-/-} mice, there are biologic consequences for enhanced IL-4R

Figure 8. STIP1 homology and U-Box containing protein 1 (STUB1) is up-regulated in airway epithelium of patients with asthma and lung tissues of patients with chronic obstructive pulmonary disease (COPD). (A) Nasal respiratory cells were collected from children or adults with asthma and corresponding normal control subjects. Total RNA was isolated, STUB1 and IL-4Rα mRNA levels were quantified using quantitative reverse-transcriptase polymerase chain reaction. (B) Total RNA was isolated from lung tissues from subjects with COPD (FEV1 ≥ 80% predicted) and normal control subjects. IL-4Rα and STUB1 mRNA levels were quantified using quantitative reverse-transcriptase polymerase chain reaction. Data are mean ± SD; **P* < 0.05.

signaling, which include lung inflammation with mucous hypersecretion and increase in serum IgE levels. Fourth, it confirms a biologic link between STUB1 and inflammatory airway diseases by revealing increased level of STUB1 in children and adults with asthma and patients with COPD.

Although much is known about initiation of cytokine signaling, the mechanisms governing cytokine signal termination are less understood. Turnover of cytokine receptors is one such mechanism and ubiquitin-proteasomal degradation of receptors represents an important pathway for their turnover (36–38). Understanding the mechanism underlying cytokine receptor turnover is critical because the levels of receptors determine the signaling threshold (39, 40). The roles of specific ubiquitin E3 ligases, such as RNF41 and FBXL19, in regulating cytokine receptor turnover and signaling have been emphasized (28, 29, 41). Despite the important role of IL-4R signaling pathway on Th2 immune response and inflammation, little is known about the molecular mechanism that controls IL-4Rα turnover or signal termination. In this study, we revealed that the steady-state level of IL-4Rα is tightly controlled by STUB1, an E3 ubiquitin ligase, which interacts with IL-4Rα and mediates IL-4Rα ubiquitination and degradation. The

biologic significance of STUB1 in IL-4Rα signaling is underscored by the observations that STUB1 deficiency sustained STAT6 activation and therefore induced airway inflammation, IgE production, and macrophage alternative (M2) activation in mice. STUB1 is also involved in the internalization and post-translational modification of IL-4Rα after ligands engagement. We found that after binding of IL-4 or IL-13 to IL-4Rα, cell surface levels of IL-4Rα decreased and IL-4Rα was targeted for STUB1-mediated ubiquitination and degradation, similar to those previous observations on other cytokine receptors, such as ST2L, IL-2Rβ, and IL-5R (29, 36, 38). These results together suggest that STUB1 is required for restraining of IL-4Rα signaling and airway inflammation.

Consistent with the interaction between STUB1 and its substrates (17, 27), we found that TPR chaperon-binding domain of STUB1 is required for its association with IL-4Rα. STUB1-K30A, the chaperon-binding domain mutant that is unable to bind to Hsp70, failed to interact with and degrade IL-4Rα, implying that the interaction between STUB1 and IL-4Rα is indirect and mediated by Hsp70. The U-box domain mutant STUB1-H260Q, which is deficient in E3 ligase activity, showed more abundant binding with IL-4Rα, suggesting that ubiquitination of IL-4Rα by

STUB1 might be required for termination of the interaction between STUB1 and IL-4Rα.

Pulmonary accumulation of inflammatory cells is the hallmark of airway inflammation. Macrophages are the most predominant immune cell population in normal lung tissue and play critical roles in the progression of airway inflammation (42). Recent studies have established the correlation between the type of macrophage activation and the inflammatory responses (31, 43). Classically activated macrophages, designated as M1 macrophages, are induced by Th1 cytokine IFN-γ, whereas the alternative M2 macrophages are activated by Th2 cytokines IL-4 and IL-13 through IL-4Rα–STAT6 pathway. Differentiation and recruitment of IL-4- and IL-13-activated M2 macrophages are required for inflammatory airway diseases, such as asthma and COPD (42, 44). In our study, we found that macrophages adopted an M2 phenotype in lungs of *STUB1*^{−/−} mice, suggesting that M2 macrophages were activated by enhanced IL-4R signaling and then mediated the lung inflammation noted in these mice. The increase of STUB1 mRNA in human asthma confirms the previous findings about the biologic associations between STUB1 and the asthmatic phenotype. IFN-γ plays an important role in inflammation in COPD and its levels are markedly increased in patients with COPD compared with healthy subjects (45, 46). Treatment with Toll-like receptor ligand LPS stimulates the production of IFN-γ (47) and is often used to induce inflammatory airway responses mimicking characteristics of COPD in animals (48, 49). In our study, we found that both IFN-γ and LPS increased IL-4Rα levels in human airway bronchial epithelial cells. Furthermore, IL-4Rα protein levels were elevated in early stage of lung tissues from subjects with COPD, suggesting the involvement of IL-4R signaling pathway in COPD pathogenesis. As a common receptor subunit shared by IL-4 and IL-13, IL-4Rα is essential for signal transduction by both cytokines. Genetic polymorphism analysis showed that IL-13 single nucleotide polymorphisms are positively associated with COPD susceptibility (50–52). Overexpression of IL-13 in murine lung caused emphysema, mucus overproduction, and airway inflammation, which are reminiscent of human COPD (53). Furthermore, disruption of IL-13 gene

prevented emphysema (54). However, IL-4 is not genetically associated with COPD (51, 52) and patients with COPD usually lack high titer of IgE in serum, whose production is dependent on IL-4 signaling. The increase of STUB1 in human asthma and COPD confirms the biologic associations between STUB1 and the airway inflammation. It might also reflect a feedback mechanism by which up-regulation of STUB1 is used to down-regulate IL-4R signaling.

Recently, STUB1 has been shown to interact with and ubiquitinate several proteins that are implicated in the pathogenesis of airway inflammation, such as Caspase recruitment domain containing membrane-associated guanylate kinase protein 1 (55), macrophage migration inhibitory factor (MIF) (56), and hypoxia-inducible factor 1- α (57). Mice with deletion of Caspase recruitment domain containing membrane-associated guanylate kinase protein 1, a lymphocyte-specific scaffold protein necessary for T-cell activation, failed to develop airway inflammation in a murine model of asthma

(58, 59). Expression level of MIF is elevated in serum and the bronchial alveoli of patients with asthma and deficiency of MIF prevents development of allergic airway inflammation in mouse model (60). Hypoxia-inducible factor 1- α is increased after challenge in patients with asthma and contributes to allergic immune responses and lung eosinophilia in a mouse model of asthma (61, 62). STUB1 has been reported to mediate ubiquitination of SMAD1 (63), an important component of BMP-SMD signaling, which is activated and up-regulated in human asthmatic airways (64). STUB1 also recruits and ubiquitinates Src and PKC ζ and then promotes Toll-like receptor signaling (65), which is critical for inflammation and immunity in the airways (66). It will be interesting to examine whether these proteins are up-regulated in *STUB1*^{-/-} mice. Thus, multiple mechanisms could contribute to the spontaneous airway inflammation observed in *STUB1*^{-/-} mice.

In conclusion, our results identified a previously uncharacterized role of STUB1 in regulating IL-4R signaling in airway

inflammation. STUB1 interacts with and regulates IL-4R α by mediating its degradation, thus influencing the IL-4R α -STAT6 signaling. Although targeting IL-4R α has been a promising strategy for asthma treatment, most current treatments focus on blocking IL-4R α activity or expression by recombinant human IL-4 variant, anti-IL-4R α mAb, and antisense oligonucleotides against IL-4R α (12). Identifying the underlying mechanism for IL-4R α degradation by STUB1 in this study might provide a new strategy for attenuating the IL-4R α -STAT6 signaling pathway by enhancing IL-4R α turnover. ■

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