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The Journal of Immunology

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J Immunol published online 17 September 2018
http://www.jimmunol.org/content/early/2018/09/14/jimmunol.1800147

The Journal of Immunology is published twice each month by The American Association of Immunologists, Inc., 1451 Rockville Pike, Suite 650, Rockville, MD 20852

Print ISSN: 0022-1767 Online ISSN: 1550-6606.

Published September 17, 2018, doi:10.4049/jimmunol.1800147

IL-17A Attenuates IFN-λ Expression by Inducing Suppressor of Cytokine Signaling Expression in Airway Epithelium

Mitsuru Niwa,* Tomoyuki Fujisawa,* Kazutaka Mori,† Katsumasa Yamanaka,‡ Hideki Yasui,* Yuzo Suzuki,* Masato Karayama,* Hironao Hozumi,* Kazuki Furuhashi,*,‡ Noriyuki Enomoto,* Yutaro Nakamura,* Naoki Inui,§ Tetsuro Suzuki,¶ Masato Maekawa,‡ and Takafumi Suda*

IFN-λ is a cytokine expressed in epithelial tissues and plays a central role in antiviral mucosal immune response. The expression of IFN-λ in the airway is impaired in chronic airway diseases (e.g., asthma, chronic obstructive pulmonary disease), which renders patients susceptible to viral infection. IL-17A is associated with asthma and chronic obstructive pulmonary disease pathogenesis; however, IL-17A regulation of IFN-λ expression remains unclear. The aim of the current study is to clarify IL-17A–mediated regulatory mechanisms of IFN-λ expression in human airway epithelial cells. In this study, we have shown that polyinosinic:polycytidylic acid (polyI:C) and influenza A virus (IAV) infection increased IFN-λ expression at mRNA and protein levels in primary cultures of normal human bronchial epithelial cells, whereas IL-17A attenuated polyI:C- or IAV-induced IFN-λ expression. IFN-λ receptor 1 knockdown and a JAK inhibitor, ruxolitinib, attenuated polyI:C-induced IFN-λ expression, confirming that a positive autocrine feedback loop, the IFN-λ receptor–JAK–STAT pathway, was involved in IFN-λ expression. In Western blotting analysis, we demonstrated that polyI:C and IAV infection induced STAT1 phosphorylation in normal human bronchial epithelial cells, whereas IL-17A suppressed polyI:C- or IAV-mediated STAT1 phosphorylation. Furthermore, we found that cotreatment with IL-17A and polyI:C or IAV infection synergistically increased suppressor of cytokine signaling (SOCS)1 and SOCS3 expression. SOCS1 small interfering RNA and SOCS3 small interfering RNA negated the inhibitory effect of IL-17A in polyI:C-induced IFN-λ expression by restoring attenuated STAT1 phosphorylation. Taken together, these findings indicate that IL-17A attenuates virus-induced IFN-λ expression by enhancing SOCS1 and SOCS3 expression to inhibit autocrine signaling loops in human airway epithelial cells. ***The Journal of Immunology*****, 2018, 201: 000–000.**

The airway epithelium of the lungs directly contacts the external environment and is subjected to dynamic physical forces. Airway epithelial cells function not only as a physical barrier but also as a regulator of innate and adaptive immune responses against microorganisms through the production of cytokines, chemokines, and antimicrobial peptides (1–7).

During viral infection, the IFN production and signaling pathway is a critical antiviral host response (8, 9). IFNs consist of three types of cytokines: type I IFNs include IFN-α and IFN-β; type II IFN constitutes IFN-γ; and type III IFNs include three members in humans, IFN-λ1–3, which are also known as IL-29, IL-28A, and IL-28B, respectively. The broad effects of IFN are explained via induction of hundreds of IFN-stimulated genes (ISGs) that inhibit viral replication, degrade viral nucleic acids, and induce viral resistance to neighboring cells (10). Among the IFN family of cytokines, IFN-λ is the only class whose activity is exerted on epithelial cells and regulates mucosal immune response to viral infection (11, 12). IFN-λs share a common cellular receptor consisting of two subunits: IFN-λ receptor (IFNLR) 1 and IL-10RB. The IL-10RB subunit is ubiquitously expressed in many cell types (13), whereas IFNLR1 is mainly expressed on epithelial cells (9). The expression of IFN-λs is induced in a variety of cell types by pattern recognition receptors including TLRs (11, 14). In particular, recent studies have demonstrated that airway epithelial cells produce IFN-λ during viral infection (15, 16). Once secreted, IFN-λs act in autocrine and paracrine manners through binding cell surface receptors, which activate JAK 1 and tyrosine kinase (Tyk) 2, as well as phosphorylation of STAT 1 and STAT2, inducing the subsequent induction of hundreds of ISGs (17–20).

The expression of IFN-λ in airway epithelial cells is impaired in subjects with chronic airway diseases, such as asthma (21–23) and chronic obstructive pulmonary disease (COPD) (24), resulting in prolonged viral infection. The bronchial epithelial cells from

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Received for publication January 31, 2018. Accepted for publication August 18, 2018.

This work was partially supported by Grant-in-Aid for Scientific Research C 26461186 (to T.F.) from the Japan Society for the Promotion of Science (JSPS) and JSPS KAKENHI Grant JP26461186.

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The online version of this article contains supplemental material.

Abbreviations used in this article: ALI, air–liquid interface; BEGM, bronchial epithelial growth medium; COPD, chronic obstructive pulmonary disease; IAV, influenza A virus; IFNLR, IFN-λ receptor; IRF, IFN regulatory factor; ISG, IFN-stimulated gene; NHBE, normal human bronchial epithelial; polyI:C, polyinosinic:polycytidylic acid; qRT-PCR, quantitative RT-PCR; siRNA, small interfering RNA; SOCS, suppressor of cytokine signaling.

patients with asthma show deficient IFN-λ protein compared with controls, which negatively correlates with virus replication (23). The bronchial epithelial cells from patients with severe therapy-resistant asthma exhibit impaired IFN-λ induction in response to rhinovirus and TLR3 ligand polyinosinic:polycytidylic acid (polyI:C) stimulation (25). Similarly, in patients with COPD, production of IFN-λ by bronchoalveolar lavage cells stimulated with rhinovirus was lower as compared with controls (24).

IL-17A is a proinflammatory cytokine that is mainly produced by Th17 cells and γδ T cells and possesses a diverse array of functions ranging from neutrophil recruitment to induction of wound repair and tissue remodeling (26). IL-17A plays an important role in the pathogenesis of chronic airway diseases, asthma (27–29), and COPD (30). High levels of IL-17A were found in induced sputum and bronchial biopsies obtained from patients with severe asthma (31, 32), and IL-17A is associated with neutrophilic airway inflammation and steroid insensitivity in patients with severe asthma (28). Moreover, IL-17A expression is elevated in the lung tissue of patients with severe COPD and correlates with lung function decline (33), indicating a role in COPD pathogenesis.

The detailed regulatory mechanisms that govern attenuation of airway IFN-λ expression in chronic airway diseases are poorly understood. The majority of studies demonstrating the impairment of IFN-λ expression in asthma or COPD did not identify the molecular mechanisms that underlie attenuated expression of IFN-λ. Thus, given that IL-17A is associated with the pathogenesis of severe asthma (28, 32) and COPD (30), to determine whether IL-17A could modulate IFN-λ expression in the airway epithelium, we investigated, in the current study, the molecular mechanisms underlying the IFN-λ expression induced by TLR3 ligand and the inhibitory effects of IL-17A on IFN-λ expression in the airway epithelium.

Materials and Methods

Cell culture

Primary normal human bronchial epithelial (NHBE) cells were purchased from Lonza (catalog no. CC-2541; Basel, Switzerland) and seeded in six-well plates at 1.8×10^4 cells/cm^2. NHBE cells in a submerged condition were cultured in commercially available bronchial epithelial growth medium (BEGM; Lonza) and incubated at 37°C in a humidified atmosphere with 5% CO_2. Conditioned media were collected from the cultured NHBE cells and stored at −80°C for immunoassays. Air–liquid interface (ALI) culture conditions were also used in some experiments, as previously described (29). NHBE cells in ALI condition were cultured in bronchial ALI medium (CloneticsB-ALI medium; Lonza).

TLR ligands and cytokine treatments

When NHBE cells reached 80–90% confluence, they were stimulated with various concentrations of polyI:C and/or IL-17A for various times. PolyI:C was purchased from Sigma-Aldrich (St. Louis, MO). Recombinant human IL-17A was from R&D Systems (Minneapolis, MN). Concentrations of polyI:C and IL-17A used in this study were 0.5–10 μg/ml and 1–50 ng/ml, respectively. For IL-17RA–neutralizing studies, a monoclonal anti-human IL-17RA Ab or mouse IgG1 isotype control (R&D Systems) was used at a concentration of 10 μg/ml.

RNA isolation and real-time PCR

Total RNA was isolated using the TRIzol reagent (Invitrogen, Carlsbad, CA). Preparation of first-strand cDNA was performed using the ReverTra Ace qPCR RT Master Mix (TOYOBO, Osaka, Japan) from 2 μg of total RNA. Quantitative RT-PCR (qRT-PCR) was also conducted using the THUNDERBIRD SYBR qPCR Mix (TOYOBO) on the 7500 FAST Detection System (Applied Biosystems, Foster City, CA) according to manufacturer instruction as described previously (7, 27, 29). The expression was normalized using human β-actin. The results were presented as $2^{-(\text{Ct of gene of interest} - \text{Ct of β-actin})}$ in arbitrary units. The list of primers used in qRT-PCR analysis is given in Table I.

ELISA

To determine the concentration of IFN-λ protein in the supernatant of the cell culture, double-sandwich ELISAs for human IFN-λ1/3 were performed using a DuoSet ELISA kit (R&D Systems). The assay sensitivity was 62.5 pg/ml. Absorbance was read at 450 nm with wavelength correction at 540 nm using a microplate reader (Synergy H1; BioTek Instruments, Winooski, VT).

Influenza A virus infection

The influenza A virus (IAV) strain, A/Yokohama/110/2009 (H3N2), was provided by Dr. Kawakami (Yokohama City Institute of Health, Japan). The median tissue culture infectious dose of viral stock solution was 6×10^5. The viral stock solution was diluted with BEGM up to 100-fold. NHBE cells cultured in six-well plates were infected with 1000 μl IAV solution for 1 h. Then, IAV was removed, the cells were washed twice with Dulbecco PBS, and the medium was replaced with complete medium. Cells were harvested at various time points.

Small interfering RNA and transient transfection of NHBE cells

The small interfering RNA (siRNA) for IFN regulatory factor (IRF) 3, IFNLR1, and suppressor of cytokine signaling (SOCS)1 were purchased from Santa Cruz Biotechnology (Dallas, TX). NF-κB p65 siRNA and random oligomer as a negative control were obtained from Sigma-Aldrich. SOCS3 siRNA was purchased from Dharmacon (Lafayette, CO). When NHBE cells reached 40–50% confluence, the medium was replaced with Opti-MEM, and cells were transfected using a transfection additive (DharmaFECT 1; Dharmacon). After 24 h of transfection, the transfection mix was exchanged to BEGM, and the cells were incubated until subconfluent. Then, cells were stimulated and harvested.

Inhibitor treatments

NHBE cells were preincubated with 0.01–1 μM JAK inhibitor (ruxolitinib; Selleck Chemicals, Houston, TX) for 1 h. During polyI:C stimulation, ruxolitinib was added at the pretreated concentration.

Western blot analysis

Total protein lysates from different treatments were harvested using RIPA lysis buffer (ATTO, Tokyo, Japan). The cell lysates were separated using a Mini-PROTEAN TGX gel (Bio-Rad Laboratories, Hercules, CA), transferred electronically to polyvinylidene fluoride membranes, and probed with the indicated Abs as described previously (6). The images were acquired using a ChemiDoc (Bio-Rad Laboratories).

Phospho-IRF3 rabbit mAb, IRF3 rabbit mAb, phospho-IκBα mouse mAb, IκBα mouse mAb, phospho-STAT1 rabbit mAb, STAT1 rabbit mAb, SOCS1 rabbit mAb, and SOCS3 rabbit mAb were purchased from Cell Signaling Technology (Boston, MA). Anti–β-actin mouse mAb was purchased from Sigma-Aldrich, HRP-conjugated goat anti-mouse IgG (W492B) was purchased from Promega (Tokyo, Japan), and HRP-linked anti-rabbit goat IgG (no. 7074) was purchased from Cell Signaling Technology. The Abs were diluted according to manufacturer description.

Statistical analysis

Measurements are described as the mean ± SE. Comparison between groups was made using the Student t test. The p values ≤ 0.05 were considered statistically significant.

Results

Induction of IFN-λ expression by polyI:C and IAV infection and inhibitory effect of IL-17A on IFN-λ expression in primary NHBE cells

To investigate the innate immune responses induced by viral respiratory infection in airway epithelial cells, primary NHBE cells in submerged cultures were challenged with different amounts of polyI:C, which is a synthetic dsRNA analogue and ligand of TLR3, for 24 h. The mRNA and protein level of IFN-λ was measured by qRT-PCR and ELISA, respectively. The mRNA expression of IFN-λ1 and IFN-λ2/3 was upregulated by polyI:C treatment in a concentration-dependent manner (Fig. 1A, 1B). ELISA demonstrated a similar increase and trend of IFN-λ protein induction in NHBE cells in a submerged condition after 24 h of exposure to polyI:C (Fig. 1C). The effect of IL-17A on IFN-λ expression in NHBE cells was then investigated by costimulating cells with polyI:C and IL-17A, followed by evaluation of IFN-λ expression.

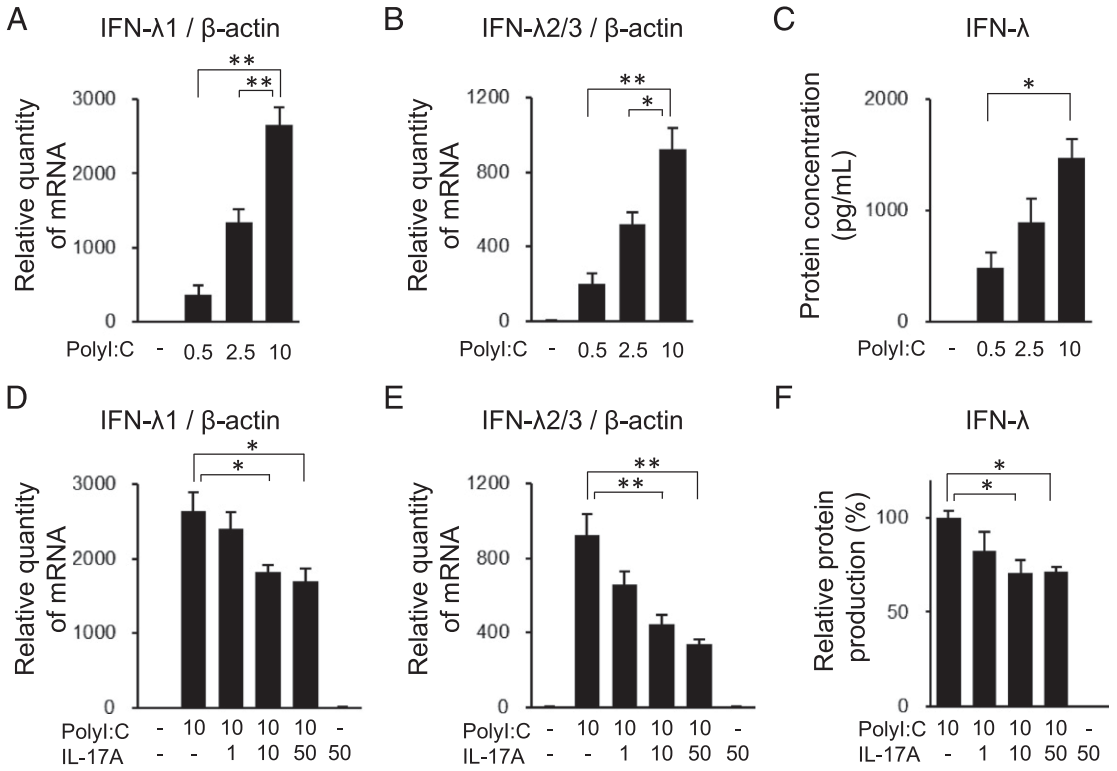

FIGURE 1. Effect of IL-17A on IFN-λ expression induced by a TLR3 ligand. NHBE cells were treated with polyI:C (0.5–10 μg/ml) and/or IL-17A (1–50 ng/ml) for 24 h. mRNA expression of IFN-λ1 (**A** and **D**) and IFN-λ2/3 (**B** and **E**) as determined by qRT-PCR and normalized to β-actin ($n = 4$). (**C**) IFN-λ protein expression as measured by ELISA in the culture supernatants ($n = 3$). (**F**) Relative expression level of IFN-λ protein at indicated conditions as compared with that of polyI:C (10 μg/ml) treatment. $n = 3$. $^*p < 0.05$, $^{**}p < 0.01$.

Notably, IL-17A treatment significantly attenuated polyI:C-induced mRNA expression of IFN-λ1 and IFN-λ2/3 in a dose-dependent manner (Fig. 1D, 1E). Similarly, polyI:C-induced IFN-λ protein was attenuated by IL-17A (Fig. 1F).

A time-course study indicated that the induction of polyI:C-mediated mRNA expression of IFN-λ1 and IFN-λ2/3 occurred from 2 to 48 h (Fig. 2A, 2B). Conversely, the inhibitory effects of IL-17A on polyI:C-induced IFN-λ1 and IFN-λ2/3 expression were first observed 24 h after treatment and continued until 48 h (Fig. 2A, 2B).

Next, NHBE cells were cultured for 7 d after transferring to ALI and then stimulated with polyI:C (10 μg/ml) and/or IL-17A (50 ng/ml) for 24 h. In well-differentiated NHBE cells cultured under ALI conditions, IL-17A significantly decreased polyI:C-induced IFN-λ protein expression in both apical and basolateral conditioned media after 24 h of treatment (Fig. 2C, 2D).

In addition, we confirmed the inhibitory effects of IL-17A in an IAV infection model. The mRNA expression of IFN-λ1 and IFN-λ2/3 was upregulated by IAV infection (Fig. 3A, 3B). IL-17A significantly attenuated the IAV infection–induced mRNA expression of IFN-λ1 and IFN-λ2/3 (Fig. 3A, 3B). IAV infection–induced IFN-λ production was attenuated by IL-17A (Fig. 3C).

Both NF-κB and IRF3 pathways are required for polyI:C-induced IFN-λ expression

We have previously demonstrated that a diverse pathway including the IRF3 and NF-κB pathways mediated epithelial proinflammatory cytokine production by polyI:C (6, 7). To confirm the involvement of IRF3 and NF-κB in polyI:C-mediated IFN-λ expression, we used the siRNA approach to knockdown IRF3 or p65 NF-κB in NHBE cells. The efficiency of siRNA in reducing endogenous target mRNA was confirmed by qRT-PCR. *IRF3* mRNA expression was reduced to <20% basal level by siRNA treatment (Supplemental Fig. 1A) and that of p65 was reduced to <10% (Supplemental Fig. 1B). Both IRF3 siRNA and p65 siRNA attenuated polyI:C-induced mRNA expression of IFN-λ1 and IFN-λ2/3 from 4 to 24 h after polyI:C stimulation compared with the negative control (Supplemental Fig. 1C, 1D). These results demonstrated that both

Table I. Primers used in real-time RT-PCR analysis

Target Gene	Sequence
β-actin	Forward 5′-AGTCGGTTGGAGCGAGCAT-3′
	Reverse 5′-AAAGTCCTCGGCCACATTGT-3′
IFN-λ1 (*IL29*)	Forward 5′-GGCCCCCAAAAAGGAGTCC-3′
	Reverse 5′-GGTGTGGGGTGTCAGGTG-3′
IFN-λ2/3 (*IL28A/B*)	Forward 5′-AGTTCCGGGCCTGTATCCAG-3′
	Reverse 5′-AAGAGGTTGAAGGTGACAGAGG-3′
IRF3	Forward 5′-TTTTCCCAGCCAGACACCTC-3′
	Reverse 5′-CCAGAATGTCTTCCTGGGTATCAG-3′
p65 (*RELA*)	Forward 5′-AGCTCAAGATCTGCCGAGTG-3′
	Reverse 5′-ACATCAGCTTGCGAAAAGGA-3′
IFNLR1	Forward 5′-TTTGTGGCCTATCAGAGCTCTC-3′
	Reverse 5′-GCGTCCCTTGAACTTGTTGTAC-3′
SOCS1	Forward 5′-AACTGTATCTGGAGCCAGGAC-3′
	Reverse 5′-AACCCCTGGTTTGTGCAAAG-3′
SOCS2	Forward 5′-TCGGTCAGACAGGATGGTACTG-3′
	Reverse 5′-TGGTCCAGCTGATGTTTTAACAG-3′
SOCS3	Forward 5′-GGAGTTCCTGGACCAGTACG-3′
	Forward 5′-TTCTTGTGCTTGTGCCATGT-3′
SOCS4	Reverse 5′-AGACTGATGGCGATGGTGATG-3′
	Forward 5′-GGCACTTTCTGGATGTATCTCC-3′
SOCS5	Reverse 5′-GTGGACATGAACTCCAACAGATG-3′
	Forward 5′-GGCTTAATCCCAGTTGTAAGGC-3′
CISH	Reverse 5′-AGCCCAGACAGAGAGTGAGC-3′
	Forward 5′-TGACAGCGTGAACAGGTAGC-3′

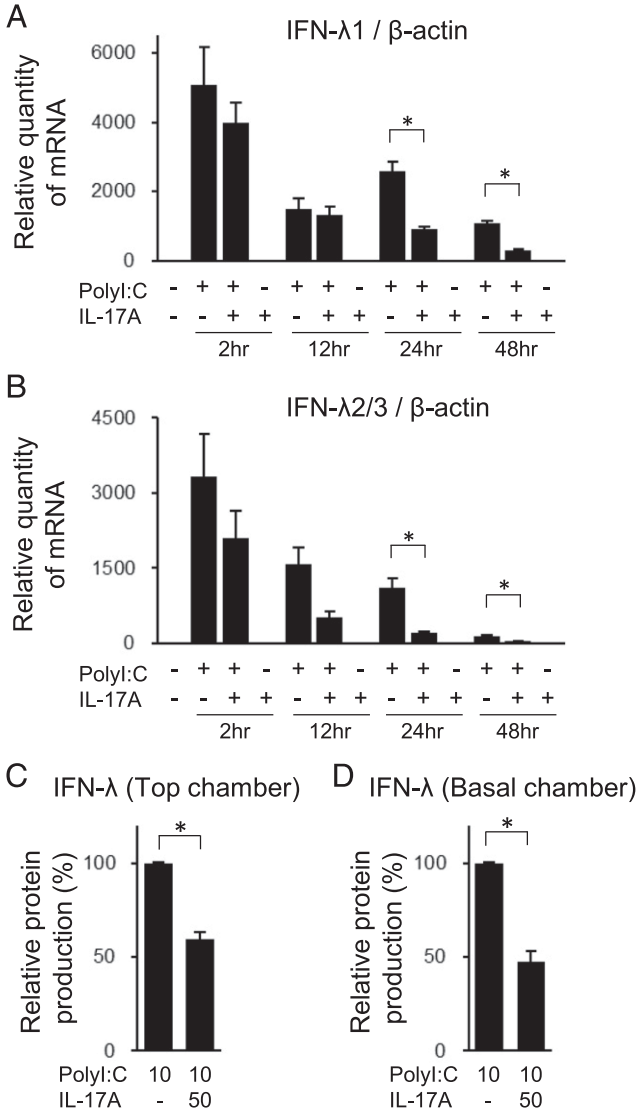

FIGURE 2. Time-course analysis of mRNA expression and protein induction with ALI culture condition. mRNA expression of IFN-λ1 (**A**) and IFN-λ2/3 (**B**) in NHBE cells at indicated times and conditions as evaluated by qRT-PCR ($n = 4$). (**C**) Relative IFN-λ protein expression level in culture supernatants of the top chamber of NHBE cells under ALI condition ($n = 3$). (**D**) Relative IFN-λ protein expression level in culture supernatants in the basal chamber of NHBE cells under ALI condition. $n = 3$. *$p < 0.05$. hr, hour.

the IRF3 and NF-κB pathways were involved in polyI:C-induced IFN-λ gene expression in airway epithelial cells.

PolyI:C-induced IFN-λ enhances its own expression in an autocrine/paracrine manner through the IFNLR1–JAK–STAT signaling axis

To further confirm a positive feedback loop of IFN-λ expression, we next investigated whether secreted IFN-λ enhanced its own expression in autocrine/paracrine manners. The receptors of IFN-λ are formed from a heterodimer of IFNLR1 and IL10R2, with the JAK–STAT pathway included in the downstream signaling. To evaluate the involvement of IFNLR1 and the JAK–STAT pathway in polyI:C-induced IFN-λ expression, an IFNLR1 siRNA and a JAK inhibitor, ruxolitinib, were used. NHBE cells were transfected with IFNLR1 siRNA, which effectively reduced *IFNLR1* mRNA expression to 20% basal levels (Fig. 4A). The IFNLR1 siRNA treatment had no effect on polyI:C-induced IFN-λ expression at 4 h, although it significantly attenuated IFN-λ expression at 24 h after polyI:C stimulation compared with the negative control (Fig. 4B, 4C). Similarly, ruxolitinib significantly decreased the mRNA expression of IFN-λ1 and IFN-λ2/3 at 24 h after stimulation in a concentration-dependent manner (Fig. 4D, 4E). These results indicated that polyI:C-induced IFN-λ expression enhances its own expression in an autocrine/paracrine manner through the IFNLR1–JAK signaling pathway.

PolyI:C treatment activates IRF3, NF-κB, and STAT1 pathways, whereas IL-17A attenuates STAT1 activation

To evaluate the intracellular signaling activated by polyI:C treatment and cotreatment with polyI:C and IL-17A, Western blotting analysis was performed. NHBE cells were stimulated with polyI:C and/or IL-17A, and the total cell lysates were harvested at 2, 6, and 24 h after stimulation. As shown in Fig. 5A, IRF3 was phosphorylated only in early phase, at 2 h after polyI:C treatment. The addition of IL-17A did not affect IRF3 phosphorylation. Phosphorylation and degradation of IκBα were detected at 2 and 24 h after polyI:C treatment, respectively; however, IL-17A had no impact on polyI:C-induced NF-κB activation (Fig. 5A). Regarding the JAK–STAT pathway, polyI:C induced STAT1 phosphorylation at 6 to 24 h but not at 2 h. Notably, the addition of IL-17A significantly attenuated polyI:C-induced STAT1 phosphorylation after 24 h of treatment (Fig. 5A, 5B). Moreover, although polyI:C induced STAT1 expression at 24 h, cotreatment with polyI:C/IL-17A had no effect on the total STAT1 protein levels (Fig. 5C).

mRNA expression of IFN-λ was induced by polyI:C treatment from early phase; however, an inhibitory effect of IL-17A appeared from 24 h after stimulation (Fig. 2A, 2B). Western blotting analysis revealed that IL-17A had no effect on polyI:C-induced activation of IRF3 and NF-κB but significantly attenuated subsequent activation of STAT1 at 24 h after treatment. These findings suggest that early activation of the NF-κB and IRF3 pathway and subsequent activation of the STAT1 pathway were all involved in polyI:C-mediated IFN-λ expression, whereas IL-17A attenuated polyI:C-mediated IFN-λ expression through suppressing the JAK–STAT1 pathway, which acts as a downstream signaling effector of the IFNLR.

Cotreatment with polyI:C and IL-17A enhances SOCS1 and SOCS3 expression

The SOCS family members are known as inhibitors of the JAK–STAT pathway. We thus next evaluated mRNA expression of SOCS family genes by polyI:C and/or IL-17A treatment. *SOCS1* mRNA expression was induced by a single treatment with polyI:C (Fig. 6A). Notably, the expression was further increased by cotreatment with polyI:C and IL-17A. Similarly, *SOCS3* expression was synergistically upregulated by cotreatment with polyI:C and IL-17A (Fig. 6C). Conversely, no induction was observed in the expression of *SOCS2*, *SOCS4*, *SOCS5*, and *CISH* (Fig. 6B, 6D–F). In Western blot analysis, cotreatment with polyI:C and IL-17A synergistically increased the protein expression of SOCS1 as compared with the single treatment (Fig. 6G, 6H). The protein expression of SOCS3 tended to be upregulated by cotreatment with polyI:C and IL-17A (Fig. 6G, 6I).

As shown in Fig. 3, IL-17A attenuated IAV infection–induced IFN-λ expression. We further evaluated SOCS expression and STAT1 phosphorylation in the IAV infection model. The mRNA expression of *SOCS1* and *SOCS3* was significantly upregulated by IAV infection with IL-17A treatment (Supplemental Fig. 2A, 2B). Moreover, the addition of IL-17A significantly attenuated IAV infection–induced STAT1 phosphorylation (Supplemental Fig. 2C).

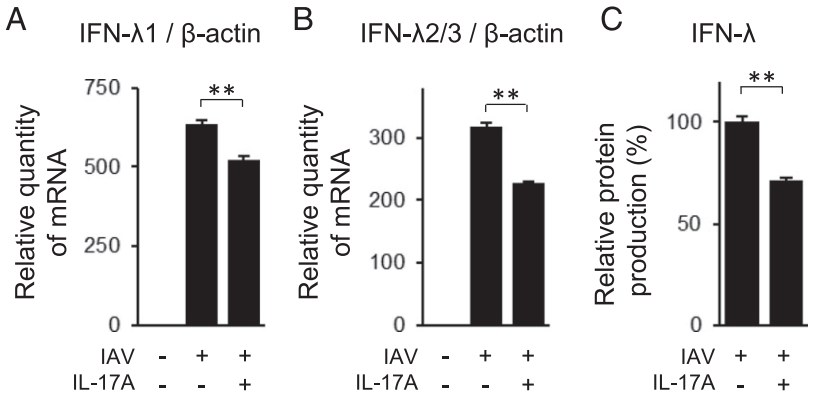

FIGURE 3. Inhibitory effects of IL-17A on IFN-λ expression in the IAV infection model. NHBE cells were infected with IAV in BEGM for 1 h, the cells were washed twice with Dulbecco PBS, and the medium was replaced with complete medium. The cells were pretreated with IL-17A (50 ng/ml) for 24 h and infected with IAV with IL-17A for 1 h. After IAV was removed and the cells were washed twice, the cells were incubated in complete medium with IL-17A (50 ng/ml). At 48 h after viral inoculation, the cells were harvested to determine mRNA expression of IFN-λ1 (**A**) and IFN-λ2/3 (**B**) by qRT-PCR and normalized to β-actin ($n = 3$). At 72 h after viral inoculation, the culture supernatants were collected to measure IFN-λ protein expression (**C**) by ELISA. $n = 4$. $^{**}p < 0.01$.

Knockdown of SOCS 1 or SOCS 3 ameliorates the inhibitory effect of IL-17A on polyI:C-induced IFN-λ expression

To evaluate the involvement of SOCS1 and SOCS3 in the attenuation of IFN-λ expression by IL-17A, we used siRNA knockdown. NHBE cells were transfected with SOCS1 or SOCS3 siRNA. The efficiency of SOCS1 siRNA in reducing endogenous *SOCS1* mRNA was confirmed by qRT-PCR (Fig. 7A). SOCS1 siRNA negated the inhibitory effect of IL-17A on polyI:C-induced IFN-λ1 and IFN-λ2/3 expression (Fig. 7B, 7C). Similarly, SOCS3 siRNA also negated the inhibitory effect of IL-17A in polyI:C-induced IFN-λ mRNA expression (Fig. 7D–F). To further determine whether SOCS1 and SOCS3 attenuated the phosphorylation of STAT1, Western blotting was performed using NHBE cells transiently transfected with SOCS1 or SOCS3 siRNA (Fig. 7G). The addition of IL-17A reduced polyI:C-mediated STAT1 phorphorylation in the negative control; however, both SOCS1 siRNA and SOCS3 siRNA restored attenuated STAT1 phosphorylation by IL-17A. These results confirm that IL-17A reduces polyI:C-mediated IFN-λ expression by enhancing SOCS1 and SOCS3 expression, which attenuates the JAK–STAT1 signaling pathway in airway epithelial cells.

Neutralization of IL-17RA abolished the inhibitory effect of IL-17A on polyI:C-induced IFN-λ expression

To determine whether IL-17A exerts inhibitory effects on polyI:C-induced IFN-λ expression through IL-17RA in NHBE cells, cultured cells were preincubated with anti-human IL-17RA Ab for

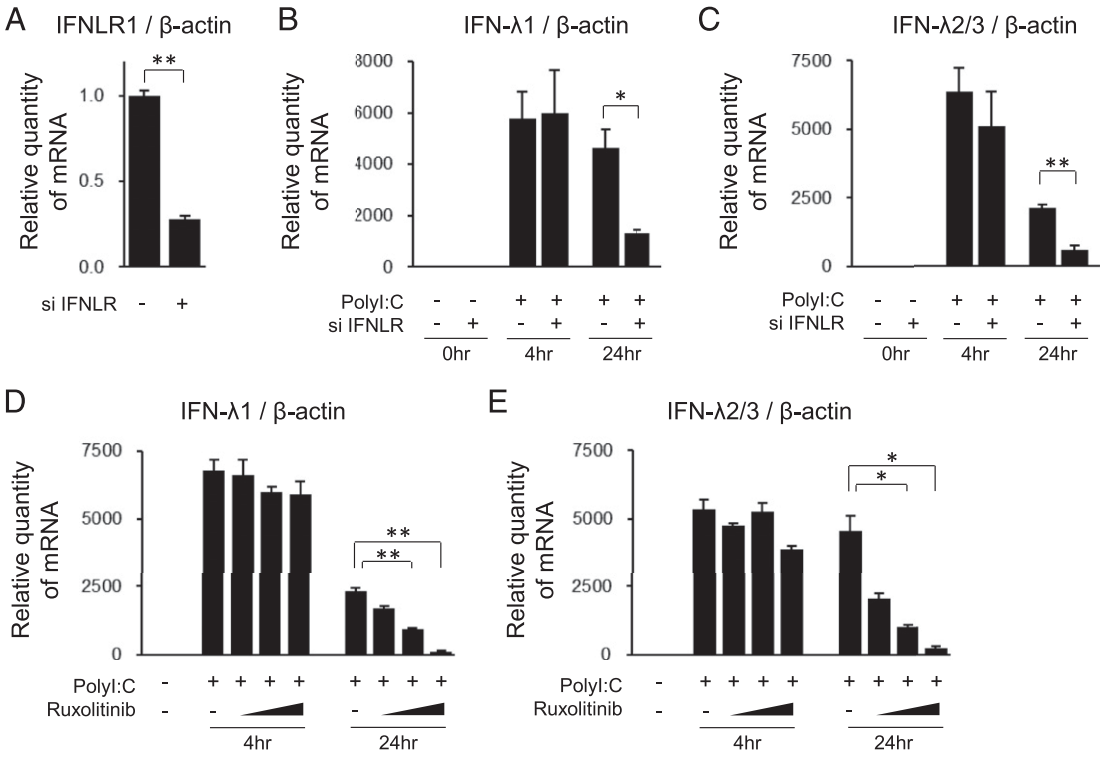

FIGURE 4. IFNLR1 and the JAK–STAT pathway are involved in polyI:C-induced IFN-λ expression. (**A**) Efficiency of IFNLR1 siRNA in reducing endogenous *IFNLR1* mRNA expression. mRNA expression of IFN-λ1 (**B**) and IFN-λ2/3 (**C**) at indicated times and conditions (IFNLR1 siRNA) ($n = 4$ each). mRNA expression of IFN-λ1 (**D**) and IFN-λ2/3 (**E**) at indicated times and ruxolitinib concentrations. $n = 3$ each. $^{*}p < 0.05$, $^{**}p < 0.01$. hr, hour.

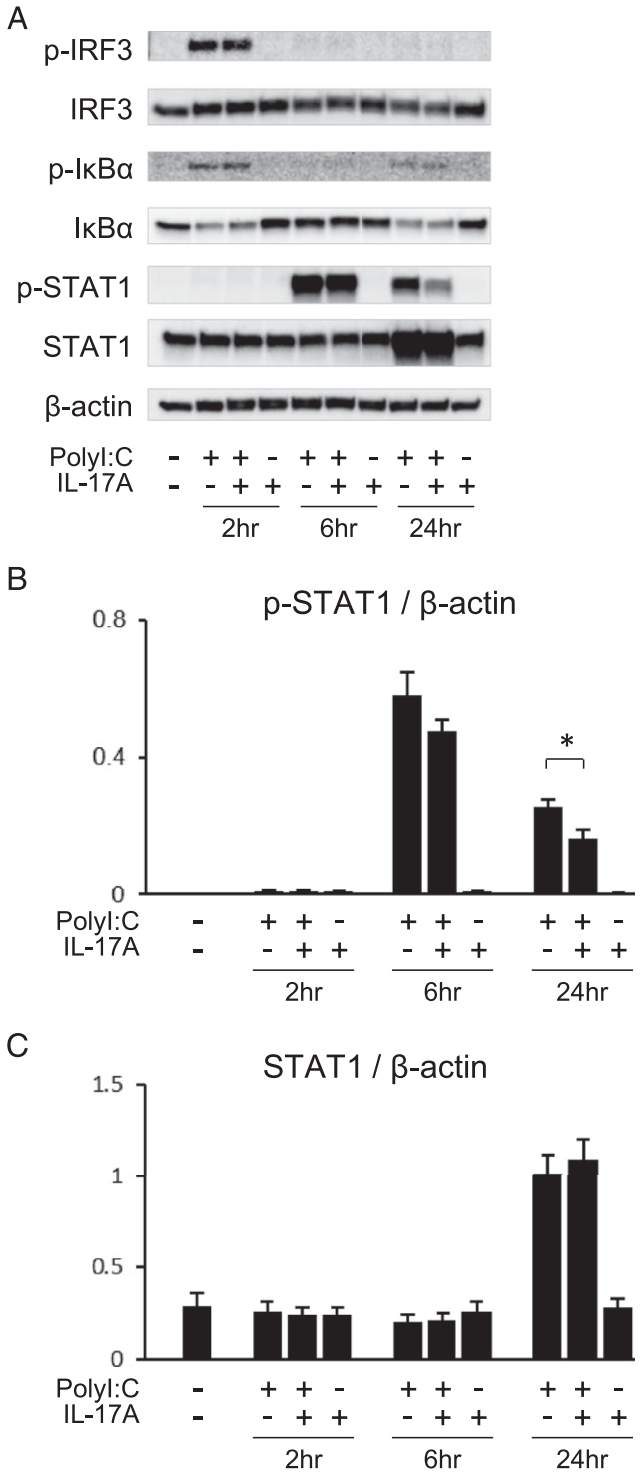

FIGURE 5. PolyI:C treatment activates IRF3, NF-κB, and STAT1 pathways, whereas IL-17A attenuates STAT1 activation. (**A**) Phosphorylation of IRF3, IκBα, and STAT1 as assessed by Western blotting. β-actin was used as a loading control. Band images from a representative experiment are shown. Semiquantification of phospho-STAT1 (**B**) and STAT1 (**C**) using densitometry. $n = 4$ each. $*p < 0.05$. hr, hour.

1 h and treated with polyI:C and/or IL-17A with/without anti-human IL-17RA Ab. At 24 h, the cells were harvested for gene expression and Western blotting analyses. The culture supernatants obtained at 48 h were used for detecting IFN-λ protein expression. Neutralization of IL-17RA partially restored polyI:C-induced IFN-λ expression attenuated by IL-17A treatment (Fig. 8A–C). Furthermore, neutralization of IL-17RA attenuated the synergistic upregulation of SOCS1 and SOCS3 mRNA by cotreatment with polyI:C and IL-17A (Fig. 8D, 8E). Western blotting analysis revealed that neutralization of IL-17RA partially restored polyI:C-mediated STAT1 phosphorylation, which was attenuated by IL-17A (Fig. 8F, 8G). These findings indicate that IL-17A attenuated polyI:C-induced IFN-λ expression, enhanced SOCS1 and SOCS3 expression, and attenuated STAT1 phosphorylation through binding to IL-17RA.

Discussion

In the current study, we investigated the molecular mechanisms underlying TLR3 ligand–induced IFN-λ expression and the inhibitory effects of IL-17A on IFN-λ expression in human airway epithelial cells. We found, for the first time to our knowledge, that IL-17A suppressed polyI:C- and IAV infection–induced IFN-λ expression in primary culture of NHBE cells. Analysis of intracellular signaling transduction revealed that both NF-κB and IRF3 activation were required for polyI:C-induced IFN-λ expression. Moreover, an autocrine positive feedback loop, the IFNLR1–JAK–STAT pathway, was involved in the persistent IFN-λ expression. Cotreatment with IL-17 and polyI:C or IAV infection synergistically increased SOCS1 and SOCS3 expression, which attenuated polyI:C- or IAV-mediated STAT1 phosphorylation and resulted in suppression of IFN-λ expression, without any impact on NF-κB and IRF3 activation. Knockdown of SOCS1 or SOCS3 expression using specific siRNA negated the inhibitory effect of IL-17A on polyI:C-induced IFN-λ expression by restoring STAT1 phosphorylation. These findings provide evidence that IL-17A exerts inhibitory action on the IFN-λ production induced by TLR3 ligand in human airway epithelial cells by inducing SOCS1 and SOCS3 expression to attenuate STAT1 activation, which may cause impaired antiviral immune response in chronic airway diseases, such as asthma and COPD.

In this study, we have shown that IL-17A suppressed polyI:C- and IAV infection–induced IFN-λ production in NHBE cells. IL-17A is a cytokine that induces the expression of a variety of cytokines, chemokines (6, 7, 34), and mucins (27, 29) in airway epithelial cells and plays a key role in the pathogenesis of asthma and COPD, including neutrophilic airway inflammation and airway remodeling (e.g., mucus metaplasia, airway fibrosis) (27, 29, 35). Former studies have shown that elevated levels of IL-17A were found in patients with severe asthma and COPD (28, 33), a group known to be more susceptible to viral infection than healthy subjects (36). In addition, neutrophilic forms of asthma are associated with IL-17A, and these endotypes are generally severe and resistant to corticosteroid treatment (37–40). Given that IFN-λ plays a central role in the mucosal host defense response against viral infection, attenuation of airway IFN-λ production by IL-17A, which constitutes a novel finding in this study, may thus represent one of the causes of defective antiviral immunity in chronic airway diseases, especially in severe neutrophilic asthma. In addition, viral infection is the most common cause of acute exacerbation of asthma (41, 42) and COPD (36) in clinical practice. Impairment of IFN-λ production would likely bring about a prolonged airway viral infection, which may provoke exacerbation of asthma and COPD. Thus, additional studies using animal models are required to determine whether elevated levels of IL-17A attenuate IFN-λ production and impair antiviral host defense response, resulting in exacerbation of asthma and COPD.

In addition, we found that not only direct activation of NF-κB and IRF3 but also positive feedback through the IFNLR–JAK–STAT1 pathway via an autocrine mechanism were involved in

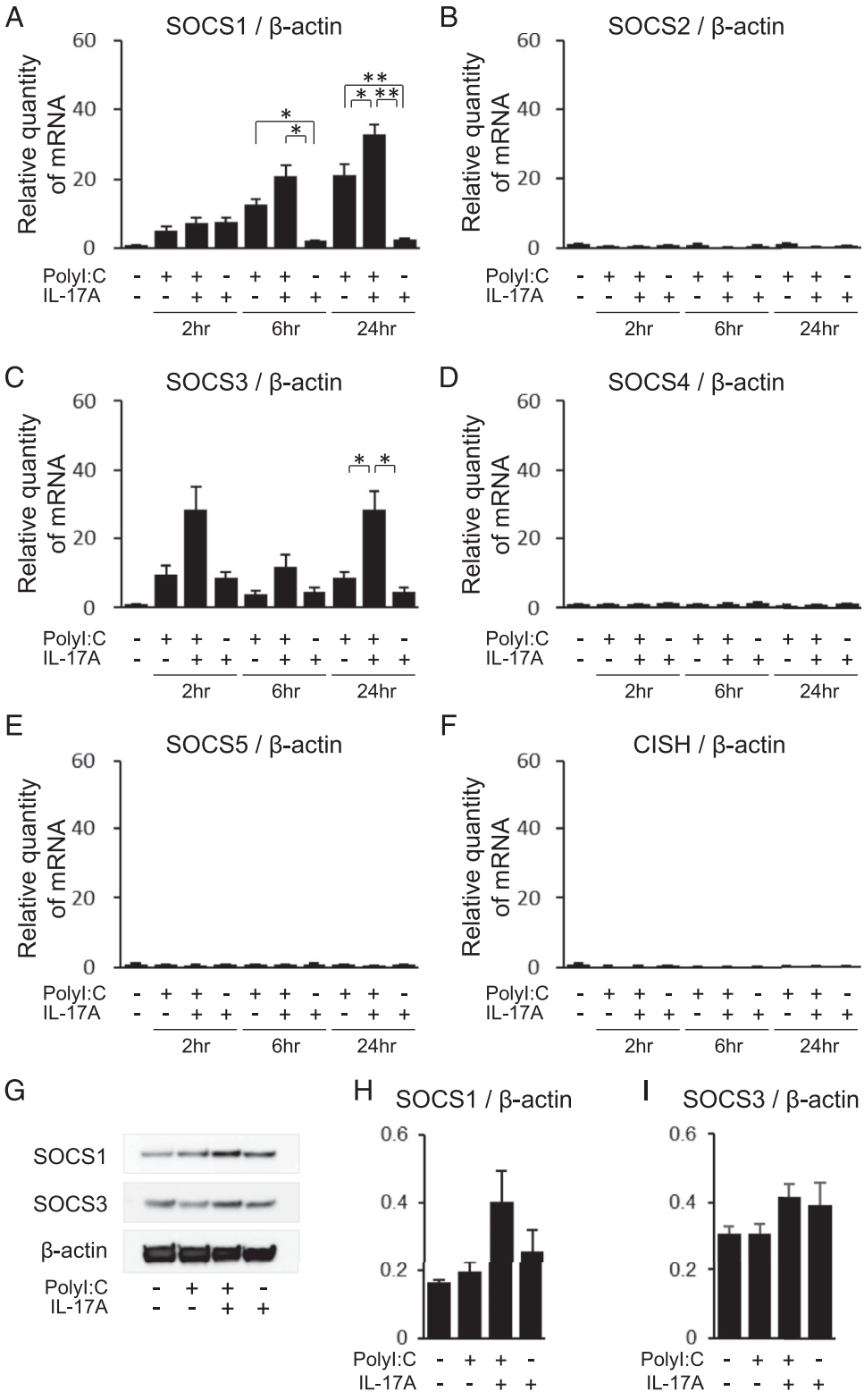

FIGURE 6. SOCS family expression by polyI:C and/or IL-17A treatments. *SOCS1* (**A**), *SOCS2* (**B**), *SOCS3* (**C**), *SOCS4* (**D**), *SOCS5* (**E**), and *CISH* (**F**) mRNA expression at indicated times and conditions. $n = 6$ (A, B, and D–F), $n = 5$ (C). (**G**) SOCS1 and SOCS3 protein expression as detected by Western blotting. Band images from a representative experiment are shown. Semiquantification of SOCS1 (**H**) and SOCS3 (**I**) using densitometry ($n = 3$ each). $^{*}p < 0.05$, $^{**}p < 0.01$. hr, hour.

polyI:C-induced IFN-λ expression. In the time-course study, induction of IFN-λ1 and IFN-λ2/3 were observed at 2–48 h after polyI:C treatment (Fig. 2A, 2B). Analysis of the signaling pathways revealed that NF-κB and IFR3 were involved in IFN-λ induction from the early stage (Supplemental Fig. 1C, 1D). Conversely, the positive feedback loop of the IFNLR–JAK–STAT1 pathway was mostly related to persistent IFN-λ expression after polyI:C treatment (Fig. 4B–E). When IFN-λ is produced in epithelial cells, IFN-λ binds to the heterodimeric receptor, IFNLR/IL10RB, leading to activation of the JAK–STAT pathway, which induces the expression of ISGs (43, 44). However, no study had been reported regarding the role of a positive autocrine/paracrine feedback loop in IFN-λ expression. The findings in this study clearly demonstrate that a positive autocrine feedback loop exists in IFN-λ expression in airway epithelial cells, which results in sustained increase of IFN-λ production and persistent antivirus immune response in airway epithelium.

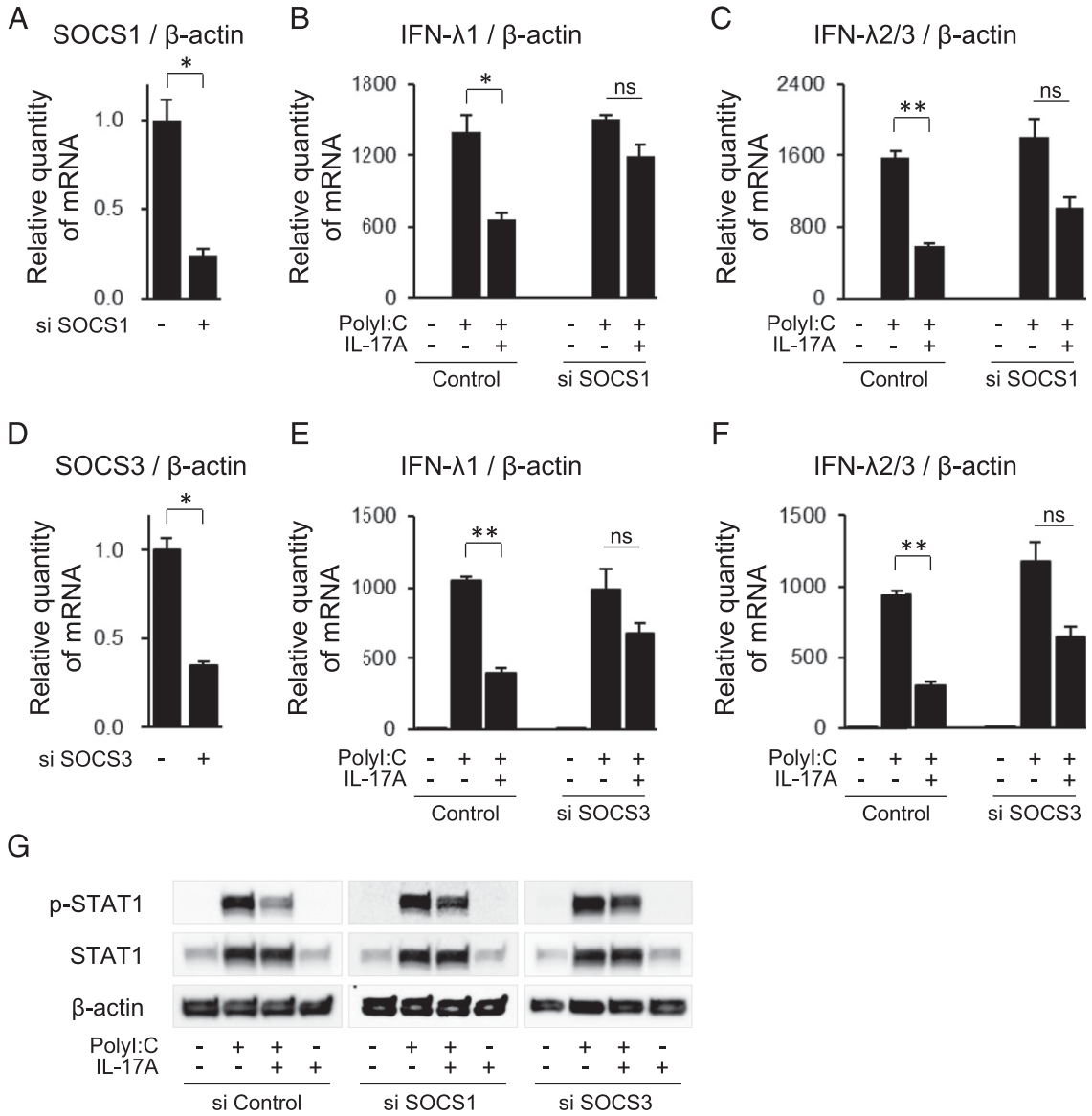

FIGURE 7. Involvement of SOCS1 and SOCS3 in the attenuation of IFN-λ expression by IL-17A. Efficiency of SOCS1 (**A**) or SOCS3 (**D**) siRNA in reducing endogenous *SOCS1* or *SOCS3* mRNA expression ($n = 3$ each). mRNA expression of IFN-λ1 (**B**) and IFN-λ2/3 (**C**) at indicated conditions (SOCS1 siRNA) at 24 h of treatment ($n = 3$ each). mRNA expression of IFN-λ1 (**E**) and IFN-λ2/3 (**F**) at indicated conditions (SOCS3 siRNA) at 24 h of treatment ($n = 3$ each). (**G**) Phosphorylation of STAT1 as assessed by Western blotting at indicated conditions at 24 h of treatment. Band images from a representative experiment are shown. $*p < 0.05$, $**p < 0.01$. ns, not significant.

Binding of IL-17A to its receptors results in the activation of NF-κB, MAPK, and C/EBPs (45). In this study, phosphorylation and degradation of IκBα were not observed at 2 and 24 h after IL-17A treatment, and IL-17A had no impact on polyI:C-induced NF-κB activation (Fig. 5A). Huang et al. (5) have reported that IL-17A treatment phosphorylated IκBα in NHBE cells within 3–10 min, and the IκBα phosphorylation returned to the control level at 60 min after IL-17A treatment. It is possible that IL-17A treatment activates the NF-κB pathway at an earlier stage in our experimental condition. Different time courses might yield different results. Considering that IL-17A did not induce IFN-λ expression and had no additive effect on polyI:C-induced IFN-λ mRNA expression, it is reasonable that IL-17A–mediated NF-κB activation is not involved in IFN-λ expression.

The members of SOCS family proteins are known as important negative regulators of JAK–STAT signaling pathways (46), which bind to the receptors of cytokines and JAKs and prevent STATs from access to the receptor kinase complex. In the current study, we have shown that cotreatment with IL-17A and polyI:C or IAV infection synergistically enhanced SOCS1 and SOCS3 expression, which attenuated the STAT1 phosphorylation mediated by the autocrine positive feedback of IFN-λ and decreased IFN-λ expression. In the context of viral infection, influenza virus induces SOCS1 and SOCS 3 expression, which suppresses virus-induced IFN-β promoter activation in the human bronchial epithelial BEAS-2B cells (47). In addition, Gielen et al. (22) demonstrated that SOCS1 expression was increased in primary bronchial epithelial cells from patients with asthma and was associated with deficiency of IFN-λ expression and increased viral replication, suggesting that SOCS1 negatively regulates IFN-λ expression. However, there are few studies regarding the molecular details of the regulation of airway IFN-λ expression by SOCS family proteins. In this study, we revealed that both SOCS1 and SOCS3 induced by cotreatment with IL-17A and polyI:C or IAV infection negatively regulated IFN-λ expression by inhibiting the JAK–STAT1 signaling pathway. As IFN-λ is critical to antiviral

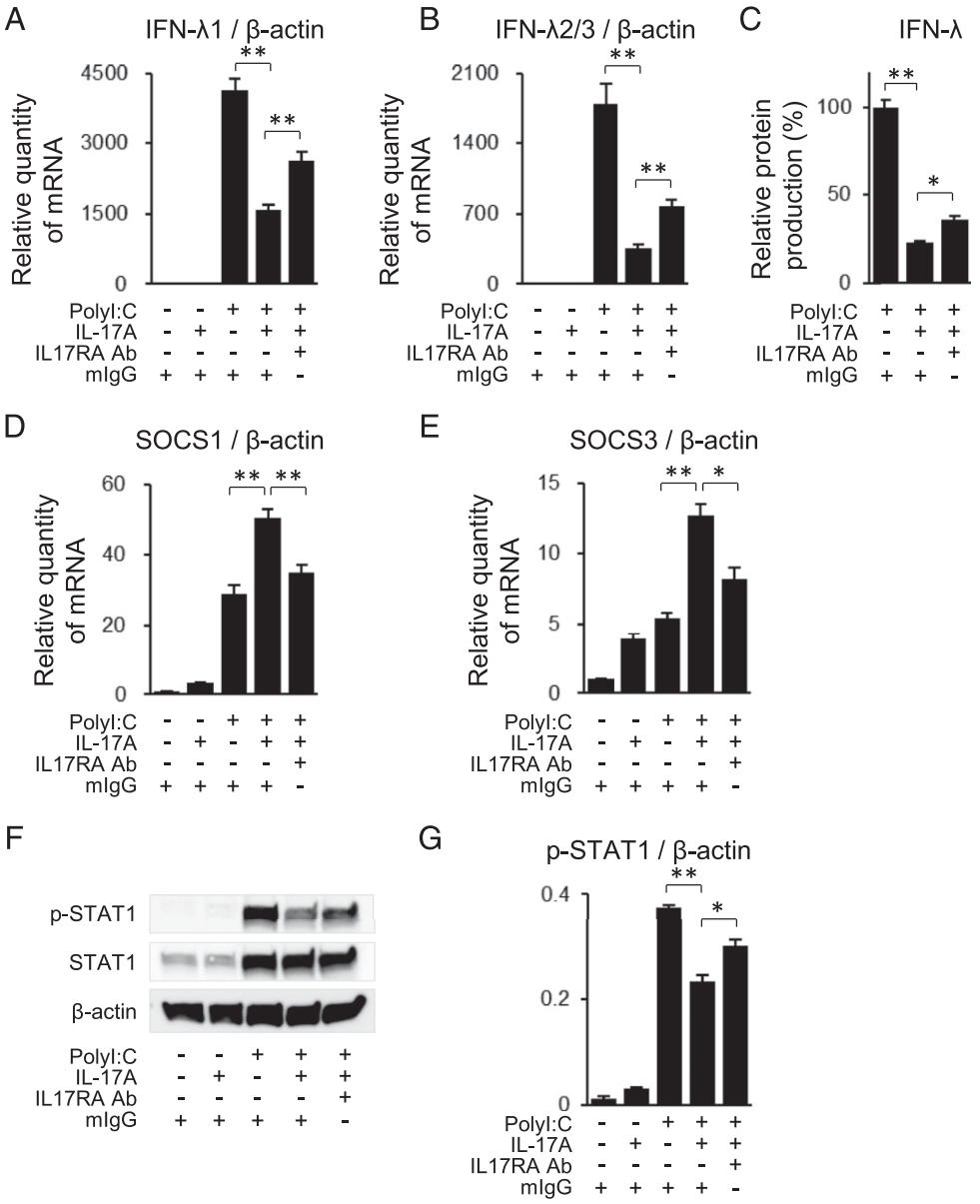

FIGURE 8. Neutralization of IL-17RA restores IFN-λ expression. NHBE cells were pretreated with anti-human IL-17R Ab for 1 h and then treated with polyI:C (10 μg/ml), IL-17A (50 ng/ml), and/or IL-17R Ab (10 μg/ml). mRNA expression of IFN-λ1 (**A**) and IFN-λ2/3 (**B**) at 24 h, as determined by qRT-PCR and normalized to β-actin ($n = 4$). (**C**) IFN-λ protein expression in the culture supernatants at 48 h, as measured by ELISA ($n = 3$). mRNA expression of SOCS1 (**D**) and SOCS3 (**E**) at 24 h of treatment ($n = 4$ each). (**F**) Phosphorylation of STAT1 at indicated conditions at 24 h of treatment, as assessed by Western blotting. Bands from a representative experiment are shown. Semiquantification of phospho-STAT1 (**G**) using densitometry ($n = 3$). $*p < 0.05$, $**p < 0.01$.

defense at epithelial surfaces, determining specific regulators of IFN-λ expression may thus lead to the development of new strategies for modulating epithelial antiviral host defense in the lung.

Notably, we identified the unique function of IL-17A to enhance TLR-mediated SOCS1 and SOCS3 expression in airway epithelial cells, which results in attenuation of IFN-λ production. IL-17A is reported to modulate epithelial response to TLR simulation and cytokines (3, 6, 48–51). Our previous study demonstrated that IL-17A synergistically enhanced polyI:C-induced proinflammatory cytokines and chemokine expression (G-CSF, IL-8, CXCL1, CXCL5, and IL-1F9) in NHBE cells (6), all of which promote neutrophil recruitment and excessive airway inflammation. The findings of the current study, that IL-17A enhances SOCS1 and SOCS3 expression resulting in attenuation of IFN-λ production, provide a new aspect of the pathogenic role of IL-17A in chronic airway diseases. Defective antiviral defense response by impaired IFN-λ expression and excessive neutrophilic airway inflammation, both of which are mediated by IL-17A, may thereby cause virus-induced exacerbation of asthma and COPD.

It remains controversial whether antiviral responses are impaired in patients with asthma. A few studies have reported that antiviral responses do not differ between asthmatics and control (52, 53). Kennedy et al. (52) reported that subjects with and without asthma infected with rhinovirus had similar viral load in their nasal washes. Patel et al. (53) demonstrated that the IFN response to IAV and rhinovirus was similar in airway epithelial cells obtained from asthmatic and nonasthmatic subjects. Recently, it was shown that the pathophysiology of asthma involves a variety of immune mechanisms (e.g., Th2 immune response, Th17 immune response) and that disease severity is divergent among patients (54). It is possible that differences in the immunological background and severity of asthma might affect the antiviral response. Based on

the findings of the current study, elevated IL-17A expression in the airway, which is sometimes observed in patients with severe asthma (31, 32), may cause attenuation of airway IFN-λ production, resulting in defective antiviral immunity. Further studies are warranted to elucidate the mechanisms of antiviral response in chronic airway diseases.

In conclusion, our data provide novel findings that IL-17A attenuates virus-induced IFN-λ expression through induction of SOCS1 and SOCS3 expression, which inhibits activation of JAK–STAT1 signaling in a positive autocrine feedback loop of IFN-λ expression in human airway epithelial cells. As airway IFN-λ plays pivotal roles in mucosal immune response to viral infection, restoring IFN-λ expression in chronic airway diseases, such as asthma and COPD, may thereby improve impaired host defense response to viral infection and prevent subsequent exacerbation. In this context, blockage of IL-17A or SOCS1 and SOCS3 expression in the airway epithelium may constitute novel therapeutic strategies to prevent viral infection and exacerbation, especially for patients with severe asthma and COPD.

Acknowledgments

We thank Terumi Taniguchi (Department of Laboratory Medicine, Hamamatsu University School of Medicine) for expert technical assistance and Dr. Takeshi Chida (Second Division, Department of Internal Medicine, Hamamatsu University School of Medicine) for technical advice.

Disclosures

The authors have no financial conflicts of interest.

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