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SOCS3 Regulates BAFF in Human Enterocytes under Ribosomal Stress

Kee Hun Do,* Hye Jin Choi,* Juil Kim,* Seong-Hwan Park,* Ki-Hyung Kim,†‡ and Yuseok Moon*†‡

Although the activation of B cells in the gastrointestinal tract is of great importance in the context of immunity to pathogens and mucosal inflammatory diseases, little is known about the mechanisms responsible for the local activation of B cells in the subepithelial area of the intestine. Epithelium-derived BAFF is the major modulator of B cell development and Ig class switching. The present study was performed to address the molecular mechanism of BAFF expression in gut epithelial cells in the presence of proinflammatory stimuli. Inflammation-induced BAFF expression in mucosal epithelial cells might be responsible for diverse mucosa-associated diseases linked to intestinal inflammation and autoimmunity. Although BAFF was marginally expressed in unstimulated epithelial cells, BAFF mRNA was significantly upregulated by proinflammatory IFN-γ. Furthermore, IFN-γ triggered JAK/STAT1 signals via the cytokine receptor, which contributed to intestinal inflammation and autoimmunity. Although BAFF was marginally expressed in unstimulated epithelial B cells, BAFF mRNA was significantly upregulated by proinflammatory IFN-γ. Furthermore, IFN-γ triggered JAK/STAT1 signals via the cytokine receptor, which contributed to intestinal inflammation and autoimmunity. In terms of signaling intervention, ribosomal insult attenuated IFN-γ–activated JAK/STAT signal transduction and subsequent BAFF induction in gut epithelial cells. Ribosomal insults led to the superinduction of SOCS3 by enhancing its mRNA stability via HuR RNA-binding protein. Upregulated SOCS3 then contributed to the blocking of the JAK/STAT-linked signal, which mediated BAFF suppression by ribosomal stress. All of these findings show that ribosomal stress–induced SOCS3 plays a novel regulatory role in epithelial BAFF production, suggesting that epithelial ribosomal dysfunction in association with SOCS3 may be a promising therapeutic point in BAFF-associated human mucosal diseases. The Journal of Immunology, 2013, 190: 6501–6510.

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Abbreviations used in this article: 15AcD, 15-acetyl deoxynivalenol; ANS, anisomycin; C1H, chromatin immunoprecipitation; CT, comparative threshold cycle; DON, deoxynivalenol; NIV, nivalenol; shRNA, short hairpin RNA.

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(26, 27). Mucosal epithelial cells located in crypt pockets potentiate their actions on naïve B cells expressing activation-induced cytokine deaminase, an enzyme responsible for Ig class switching.

Stress responses by ribosome-inactivating (ribosomal stress) agents that cause mucosal insults are etiological factors of epithelial inflammatory diseases and have been investigated in various experimental models (28–30). Specific ribosome-directed xenobiotics, such as ansisomycin (ANS), UV radiation, ricin, and a variety of sesquiterpenoid trichothecene fungal metabolites, can damage the functionality of 28S rRNA during gene translation, which leads to ribosomal stress that stimulates intracellular sentinel–signaling pathways. This process results in the expression of genes important for cellular homeostasis and genes critical for a variety of pathogenic processes involved in cell survival modulation, proliferation, and stress response (31, 32). Several epidemiological studies suggested links between ribosomal stress and human mucosal epithelial illness, including intestinal inflammatory diseases (33–35). Mechanistically, chemical ribosomal stress alters intestinal mucosal integrity by interfering with transepithelial resistance, epithelial differentiation, and nutrient absorption, the last of which is associated with anorexia and weight loss (36–39). Moreover, ribosomal stress triggers mucosal and systemic inflammation, mostly due to the enhanced production of proinflammatory mediators in immune-related cells (40–43).

Although the activation of B cells in the gastrointestinal tract is of great importance in the context of immunity to pathogens and the pathogenesis of intestinal inflammatory diseases, little is known about the mechanism of local B cell activation in intestinal mucosa. The present study was performed to address the molecular mechanism of BAFF expression in gut epithelial cells under proinflammatory stimuli. Epithelial BAFF expression may account for diverse pathologic events linked to intestinal inflammation and autoimmune diseases. The actions of mucosal ribosomal stress as a potent immune-signaling regulator of BAFF were addressed, which would provide new insight into therapeutic intervention in diseases with a BAFF-linked pathogenesis.

Materials and Methods

Cell culture condition and reagents

HCT-8 human epithelial cells obtained from human ileocecal tissues were purchased from the American Type Culture Collection (Rockville, MD). Cells were maintained in RPMI 1640 medium (supplemented with 10% [v/v] heat-inactivated FBS, 50 μM l-arginine, and 50 μg/ml streptomycin [all from Welgene, Daegu, South Korea]) in a 5% CO2 humidified incubator at 37˚C. Cell numbers were assessed by trypan blue (Sigma-Aldrich, St. Louis, MO) dye exclusion using a hemocytometer. Deyoxynivalenol (DON) with a purity of 97.6% was isolated from Fusarium graminearum (Sigma-Aldrich). Additional ribosome-inactivating stress agents, such as ANS, nivalenol (NIV), and 15-acetyl DON (15AcD), were purchased from Aldrich. Additional ribosome-inactivating stress agents, such as ANS, was dissolved in distilled water containing 0.1% BSA.

Western immunoblot analysis

Protein expression was compared by Western immunoblot analysis using rabbit polyclonal anti-human actin Ab, rabbit polyclonal anti-phosphorylated JAK2 Ab, goat polyclonal anti-human SOCS3 Ab, mouse monoclonal anti-human phosphorylated STAT1 Ab (Santa Cruz Biotechnology, Santa Cruz, CA), and anti-rabbit secondary Ab (Enzo Life Science, Plymouth Meeting, PA). Cells were washed with ice-cold phosphate buffered, lysed in boiling lysis buffer (1% [w/v] SDS, 1.0 mM sodium orthovanadate, and 10 mM Tris [pH 7.4]), and sonicated for 5 s. Lysates containing proteins were quantified using a BCA protein assay kit (Pierce, Rockford, IL). Fifty-microgram aliquots of protein were separated by Bio-Rad gel mini electrophoresis (Bio-Rad, Hercules, CA). Proteins were transferred onto polyvinylidene fluoride membranes (Amersham Biosciences, Piscataway, NJ), and blots were blocked for 1 h with 5% skim milk in TBST 0.1% and then probed with each Ab overnight at 4˚C. After washing three times with TBST, blots were incubated with horseradish-conjugated secondary Ab for 1 h and then washed with TBST three times. Proteins were detected using an ECL substrate (ELPIS Biotech, Taegon, South Korea).

Reverse transcription and conventional or real-time PCR

RNA was extracted using RiboEox (GeneAll Biotechnology), according to the manufacturer’s instructions. RNA (100 ng) from each sample was transferred to cDNA using PriboMune murine leukemia virus reverse transcriptase (Genebio, Nonsan, South Korea). The amplification was performed using G-Taq DNA polymerase (Cosmo Genetech, Seoul, South Korea) in a MyCycler thermal cycler (Bio-Rad), using the following parameters: denaturation at 95˚C for 5 min and different cycles of denaturation at 95˚C for 30 s, annealing at 58˚C for 30 s, and elongation at 72˚C for 30 s. An aliquot of each PCR product was subjected to 1.5% agarose and then gel electrophoresis and visualized by ethidium bromide staining. The forward and reverse complement PCR primers for amplification of each gene were human BAFF, 5'-CCT CAC GGT GGT GGT TTT CT-3' and 5'-AAA GGT GAG AAG CCA TGG AA-3'; human SOCS3, 5'-CCA CTT GAG TCT CCA GCT TC-3' and 5'-CAA ATG TGG CTT CCC CCT TA-3'; human STAT1, 5'-TCC GGG ATT CAG GAC AC-3' and 5'-ACC TGG GCT GTG ATG CAG ACC TGG-3'; human SOCS3, 5'-TCA GAT GTG TTC TTT-3' and 5'-CTG TGG TCA GTC CTT CC-3'. In real-time PCR, FAM was used as the fluorescent reporter dye and was conjugated to the 5' end of the probes to detect amplified cDNA in an iCycler thermal cycler (Bio-Rad), using the following parameters: denaturation at 94˚C for 2 min and 40 cycles of denaturation at 98˚C for 10 s, annealing at 59˚C for 30 s, and elongation at 72˚C for 30 s. Each sample was tested in triplicate. Relative quantification of gene expression was performed using the comparative threshold cycle (CT) method. The CT value is defined as the point at which a statistically significant increase in fluorescence has occurred. The number of PCR cycles (CT) required for FAM intensity to exceed a threshold just above background was calculated for test and reference reactions. In all experiments, GAPDH was used as the endogenous control.

Transient transfection

Cells were transfected with a mixture of plasmids using jetPRIME Polyplus transfection reagent (Polyplus, Illkirch, France), according to the manufacturer’s protocol. All transfection efficiencies were between 50 and 60%, as determined by the expression of a pMX–enhanced GFP vector. Stable transfectants were selected after 2 wk in 700 μg/ml weight was administered once via oral gavage in 0.2 ml PBS. Mice were sacrificed by anesthetizing with ether in a closed container, and large and small intestines, spleen, and Peyer’s patches were removed rapidly. After rinsing with PBS, tissues were filled with RiboEox solution (GeneAll Biotechnology, Seoul, South Korea) and immediately frozen for homogenization. Frozen tissue was homogenized using stainless steel beads under the control of TissueLyser II (both from QIAGEN, Hilden, Germany). RNA was extracted from the homogenate using Hybrid-R and RiboEox kits (GeneAll Biotechnology), according to the manufacturer’s instructions.

Structure and properties of plasmid

CMV-driven short hairpin RNA (shRNA) was constructed by inserting shRNA template into pSilencer 4.1-CMV-neo vector (Ambion, Austin, TX). SOCS3 shRNA targeted the sequence 5'-CCA AGA ACC TGC GCA TCC A-3'. An antisense HuR gene construct was kindly provided by Dr. Gorospe Myriam (National Institutes of Health, Baltimore, MD) and reconstituted into the pcDNA3.1-Hyg vector system (Invitrogen). The final product was designated pcDNA3.1-AshuR-Hyg. Human SOCS3 expression plasmid (pCDNA3-Myc-CIS3) was kindly provided by Dr. Akihiko Yoshimura (Keio University, Tokyo, Japan).
G418 (Life Technologies) and 400 µg/ml hygromycin B (Duchefa Biochemie, Haarlem, Netherlands). Transfected cells were maintained in complete media containing 350 µg/ml G418 or 200 µg/ml hygromycin B. For transfection of the luciferase reporter gene, a mixture of 2 µg firefly luciferase reporter and 0.2 µg Renilla luciferase, pRL-null vector (Promega, Madison, WI) per 2 µl CarriGene reagent (Kininova Life Sciences, Oceanside, CA) was applied to each well of a six-well culture plate. For the luciferase assay, after 12 h of transfection, cells were exposed to chemicals for an additional 24 h, lysed, and assayed using the dual-luciferase reporter assay system (Promega).

**Luciferase assay**

Cells were washed with cold PBS, lysed with passive lysis buffer (Promega), and centrifuged at 12,000 × g for 4 min. Supernatants were collected, isolated, and stored at −80˚C until assessed for luciferase activity. Luciferase activity was measured using a Model TD-20/20 dual-mode luminometer (Turner Designs, Sunnyvale, CA). Firefly luciferase activity was normalized against Renilla luciferase activity by dividing firefly luciferase activity by Renilla luciferase activity. The relative luciferase units calculated were compared.

**Flow cytometry analysis**

Trypsinized cells (2 × 10^6) were prepared and resuspended in 0.2 ml PBS. After adding 0.2 ml heat-inactivated PBS, cells were immediately fixed by the slow drop-wise addition of 1.2 ml ice-cold 70% (v/v) ethanol with gentle mixing and then held at 4˚C overnight. After blocking with 5% BSA in PBS for 30 min, cells were incubated with a buffer (3% BSA in PBS) containing a 1:200 dilution of mouse monoclonal anti-human BAFF primary Ab (PeproTech) at room temperature for 90 min and then washed repeatedly with PBS. Cells were then incubated with Alexa Fluor 546 anti-mouse IgG (H+L; Invitrogen) for 90 min at room temperature and washed thoroughly with PBS. Single-cell fluorescence was measured using a Becton Dickinson FACSCalibur (San Jose, CA). Data from 10,000 cells were collected in “list mode.”

**Bio-Plex assay**

Plasma was prepared from the blood samples obtained from mice. Whole blood was dropped into collection tubes containing EDTA. Tubes were gently inverted several times to mix well and centrifuged at 1000 × g for 15 min at 4˚C. The supernatant plasma was transferred to clean tubes, and the final supernatant was collected after centrifugation at 10,000 × g for 10 min at 4˚C. Samples were immediately frozen at −80˚C until analysis. Plasma IFN-γ concentration was measured using Bio-Plex 200 assay (Bio-Rad), according to the manufacturer’s instructions. The lower limits of quantitation were defined as 1.162 pg/ml for IFN-γ, and all measured results were analyzed using Bio-Plex Manager software version 6.1.

**Confocal microscopy**

Cells were incubated in a glass-bottom culture dish (SPL Life Sciences, Pocheon, South Korea). After treatment, cells were fixed in 4% paraformaldehyde diluted in PBS, permeabilized with 0.2% Triton X-100 in PBS for 10 min, blocked with 3% BSA in PBS for 2 h, and incubated with mouse polyclonal anti-HuR Ab (1:200; Santa Cruz Biototechnology) in buffer (3% BSA in PBS) at room temperature for 90 min. The cells were then washed with PBS, incubated with Alexa Fluor 546 goat anti-mouse IgG (H+L; Invitrogen) for 90 min at room temperature, washed in PBS, and stained with 100 ng/ml DAPI (absorbance at 405 nm) in PBS for 10 min. Confocal images were obtained using an Olympus FV1000 confocal microscope (Olympus, Tokyo, Japan) with single-line excitation (546 nm) or multi-track sequential excitation (546 and 633 nm). Images were acquired and processed using FV10-ASW software (Olympus). The intensity of signals from the selected four fields was measured using Multi Gauge software (Fujifilm, Tokyo, Japan).

**RNA–chromatin immunoprecipitation**

RNA–chromatin immunoprecipitation (ChIP) is a method that allows investigation of the DNA sequences that are to be examined. RNA-ChIP assays are performed like regular ChIP assays, but with an extra step of adding RNase inhibitor to protect RNA after cross-linking and the detection of pull-downed RNA by RT-PCR. Immunoprecipitation of protein–RNA complexes was performed using a modified ChIP protocol. Briefly, HCT-8 cells were seeded at 2.5 × 10^6/100-mm diameter dish in complete RPMI 1640 and grown for 24 h. Cells were then treated with DON or DMSO, and protein and RNA were cross-linked with 1% formaldehyde. Cytoplasmic extracts were isolated at 4˚C overnight with 5 µg of either rabbit anti-rabbit IgG (nonspecific control) or an Ab against HuR. Ab-bound complexes were precipitated using protein G–Sepharose beads, which were then washed sequentially for 5 min each with low-salt buffer, high-salt buffer, LiCl buffer, and TE buffer. Protein–RNA complexes were eluted from the beads with 250 µl elution buffer at 37˚C for 15 min. RNA in immunoprecipitated complexes was released by reversing the cross-linking at 65˚C for 4–5 h in the presence of 200 mM NaCl and 20 µg proteinase K. RNA was then extracted using RiboEX reagent (GeneAll Biotechnology) and subjected to RT-PCR.

**Statistical analysis**

Data were analyzed using SigmaStat for Windows (Jandel Scientific, San Rafael, CA). The Student t test was used to compare two groups. ANOVA was used to compare multiple groups, and the Student-Newman-Keuls method was used to make pair-wise comparisons.

**Results**

**Ribosomal insults suppress BAFF gene expression in intestinal epithelial cells**

Our basic hypothesis is that mucosal ribosomal insults alter production of B cell differentiation factor in the intestine, because the maturation of intestinal B lymphocytes and their communication with enterocytes are disrupted by mucosal ribosomal stress (44, 45). We assessed expression of BAFF as a potent modulator of B lymphocyte differentiation in a murine model after oral exposure to a mucosal ribosomal insult (Fig. 1). ANS, a representative chemical ribosomal insult, suppressed BAFF expression in a dose-dependent manner in the small (Fig. 1A) and large (Fig. 1B) intestines. The effects of ribosomal insult on BAFF expression was also assessed in cultured intestinal epithelial HCT-8 cells, which are frequently used as a human epithelial cell model of microbial infection and inflammatory diseases (46–48). Moreover, the source of HCT-8 cells, the ileocecum of the human small intestine, is one of the organs most susceptible to pathogenesis associated with ribosomal insult (49, 50). However, cultured intestinal epithelial cells produce very low levels of BAFF; thus, additional BAFF-triggering endogenous factors were required to simulate the physiologically inflamed gut environment. In the current study, IFN-γ was supplemented to activate intestinal epithelial cells to augment BAFF expression. Based on the observation that the intestinal expression of BAFF was significantly lower in IFN-γ−/− knock-out mice than in the wild types (Supplemental Fig. 1), IFN-γ−/− activated signal can be considered important for epithelial BAFF expression. In the cultured intestinal epithelial cells, IFN-γ enhanced BAFF gene expression, and maximal induction was achieved at 20 ng/ml (Fig. 2A). However, IFN-γ−/− induced BAFF expression was dose dependently attenuated by pretreating with ANS or another chemical ribosomal stress agent DON (Fig. 2B, 2C). Other ribosomal stress agents, such as NIV and 15AcD, also suppressed BAFF mRNA expression in human intestinal epithelial cell lines, including HCT-8 and HT-29 cells (Fig. 2D, 2E). HT-29 cell line is also popularly used in studies for intestinal epithelial inflammation and infection models (51, 52). Furthermore, ANS or DON dose dependently suppressed IFN-γ−/− elevated BAFF promoter activity, demonstrating the transcriptional regulation of BAFF expression by ribosomal stress (Fig. 2F, 2G). Intracellular production of BAFF protein was also measured by flow cytometry. IFN-γ enhanced BAFF protein levels, which were almost completely suppressed by chemical ribosomal insults, such as DON or ANS (Fig. 3). Taken together, these findings suggest that ribosomal insult interferes with BAFF epithelial expression in vivo and in vitro, particularly via transcriptional regulation.

**Ribosomal insults interfere with BAFF-inducing JAK/STAT signals**

IFN-γ−/− linked receptor activation is mediated by JAK1 and JAK2, which subsequently activate the transcription factor, STAT1 (53). To investigate the role of the JAK/STAT pathway in BAFF
expression, intestinal epithelial cells were treated with JAK inhibitor at the indicated doses in the presence of IFN-γ. We found that BAFF expression was dose dependently decreased by JAK inhibition (Fig. 4A). Moreover, JAK inhibition also led to suppression of BAFF promoter activity, indicating the involvement of JAK/STAT signals in the transcriptional activation of BAFF by IFN-γ (Fig. 4B). Based on an assumption that the ribosomal insult might interfere with the JAK/STAT pathway, JAK phosphorylation was assessed in the presence of chemical ribosomal insults. DON treatment decreased JAK2 activation by IFN-γ at 20 min and had a marginal effect on JAK1 phosphorylation (Fig. 4C), demonstrating that epithelial ribosomal stress suppresses the JAK2/STAT1 signal that is important for IFN-γ–induced BAFF expression.

Ribosomal insults suppress JAK2-linked BAFF expression via superinduced SOCS3 in human enterocytes

SOCS1 and SOCS3 proteins (representative regulators of the JAK/STAT-signaling pathway) were demonstrated to suppress IFN-γ–mediated cytokine gene expression (54, 55). To address whether SOCS proteins mediate the expressional suppression of BAFF by ribosomal insults, we assessed the effects of SOCS inhibition on BAFF expression using shRNA. Although SOCS1 inhibition had
no effect on BAFF induction (data not shown), the genetic ablation of SOCS3 dramatically prevented ribosomal stress-suppressed BAFF mRNA expression (Fig. 5A, 5B). Moreover, SOCS3 overexpression suppressed IFN-γ–induced BAFF expression in HCT-8 human enterocytes (Fig. 5C), which also confirms the negative regulation of IFN-γ–induced BAFF by SOCS3. To address signaling mediators directly regulated by SOCS3, BAFF-inducing JAK-linked signal was assessed. JAK2 phosphorylation was suppressed by the ribosomal stress (Fig. 4C); subsequently, phosphorylation of downstream STAT1 was also decreased (Fig. 5D). However, ablation of SOCS3 using shRNA attenuated the suppression of JAK2 and STAT1 signals, which indicates that SOCS3 negatively regulates JAK2-STAT1–linked signals that are crucial for BAFF induction.

Further assessment was performed to investigate the molecular mechanism of ribosomal insult–altered BAFF expression by examining whether ribosomal stress affects IFN-γ–induced epithelial SOCS3 mRNA expression. IFN-γ induced SOCS3 expression by its own negative-feedback loop, as previously reported (56). In the current study, although ribosomal insult or IFN-γ alone augmented SOCS3 mRNA expression, chemical ribosomal insult plus IFN-γ synergistically enhanced SOCS3 expression. In contrast, SOCS1 expression was altered slightly in the same experiments (Fig. 6A, 6B). Moreover, similar synergistic patterns were also
observed in terms of SOCS3 protein expression (Fig. 6C). To explain the superinduction of BAFF, we assessed the contribution of posttranscriptional regulation to BAFF mRNA superinduction. Ribosomal insult extended the stability of BAFF mRNA induced by IFN-γ (Fig. 7A). In addition, previous studies conducted by our group suggest that mucosal ribosomal stress plays a critical role in mRNA stabilization by triggering HuR translocation from nuclei to the cytoplasm, where it forms stable complexes with mRNAs containing AU-rich elements (57). In this study, we examined the involvement of HuR protein in SOCS3 mRNA stabilization by ribosomal stress; as expected, suppression of HuR expression retarded SOCS3 mRNA superinduction by cotreatment with IFN-γ and ribosomal insulting agent in human intestinal epithelial cells (Fig. 7B), and similar patterns of SOCS3 protein were also observed (Fig. 7C). Although only IFN-γ had marginal effects on cytosolic translocation of HuR protein, ribosomal insults plus IFN-γ significantly increased the translocation, which was mostly due to the action of ribosomal stress (Fig. 8A, 8B). Moreover,

FIGURE 5. Involvement of SOCS3 in the regulation of IFN-γ–induced BAFF expression. (A and B) HCT-8 cells expressing the control empty vector or SOCS3 shRNA-expression plasmid were treated with vehicle or 20 ng/ml IFN-γ (I) in the presence of 500 ng/ml DON (D) or 50 ng/ml ANS (A) for 24 h. The mRNA levels were measured using real-time RT-PCR. Different letters over each bar with the SD represent significant differences between two groups by unpaired matched comparisons (p < 0.05). In (B), lower gel blots indicate a representative blot using conventional RT-PCR. The panels in the box show the effects of SOCS3 shRNA on SOCS3 expression, as determined by RT-PCR. (C) Cells expressing the control empty vector or SOCS3 overexpression plasmid were treated with vehicle or 20 ng/ml IFN-γ for 24 h. The mRNA levels were measured using real-time RT-PCR. (D) Cells expressing the control empty vector or SOCS3 overexpression plasmid were treated with 20 ng/ml IFN-γ or 500 ng/ml DON for 20 min. Cellular lysates were subjected to Western blotting. All results are representative of two or three independent experiments. * p < 0.05 versus IFN-γ treatment group.

FIGURE 6. Effects of ribosomal insults on SOCS3 mRNA expression. (A and B) HCT-8 cells were treated with 20 ng/ml IFN-γ (I), 50 ng/ml ANS (A), 500 ng/ml DON (D), or combinations (I+A or I+D) for 45 min. mRNA levels were measured by real-time RT-PCR. For SOCS3 expression, different letters over bars with the SD represent significant differences between two groups as determined by unpaired matched comparisons (p < 0.05). (C) HCT-8 cells were treated with 20 ng/ml IFN-γ, 50 ng/ml ANS, or 500 ng/ml DON for 2 h. Cellular lysates were subjected to Western blotting. All results are representative of two or three independent experiments.
cotreatment with ribosomal insults and IFN-γ significantly enhanced the binding of HuR protein to the SOCS3 transcript (Fig. 8C), which can account for increased stabilization and production of SOCS3 mRNA via HuR protein. Taken together, all of these findings show that ribosomal insults suppress the induction of BAFF gene expression by blocking JAK2 phosphorylation via HuR-mediated SOCS3 stabilization in intestinal epithelial cells (Fig. 9).

**Discussion**

Because many immune mediators from various types of cells can affect intestinal BAFF expression redundantly, the effects of IFN-γR knockout on BAFF expression was not complete. However, it was confirmed that IFN-γ was one of the important positive regulators of epithelial BAFF induction. Epithelial BAFF is a critical mediator of the local activation of B cells in mucosa. Physiologically produced IFN-γ, as well as other endogenous factors, can trigger enterocytes to maintain some required levels of BAFF protein. However, if ribosomal stress decreased the production of IFN-γ, BAFF suppression would be due to decreased production of IFN-γ, regardless of SOCS3 induction by ribosomal stress. However, ribosome-inactivating stress has been known to enhance IFN-γ production, although extremely high doses of toxins can

**FIGURE 7.** Effects of ribosomal insults on SOCS3 mRNA stability. (A) HCT-8 cells were treated with 20 ng/ml IFN-γ for 45 min and then ribosomal stress agent (500 ng/ml DON or 50 ng/ml ANS) was added for the indicated time to assess effects on SOCS3 mRNA stability. The level of each mRNA was measured after transcriptional arrest by adding 5 μM of actinomycin D. *p < 0.05, versus IFN-γ treatment group. (B) Empty vector– or antisense HuR expression plasmid–transfected HCT-8 cells were cotreated with 20 ng/ml IFN-γ (I) and 500 ng/ml DON (D) for 24 h. mRNA was analyzed by real-time RT-PCR. *p < 0.05, versus empty vector–transfected group treated with IFN-γ and DON. (C) Cells expressing the control empty vector or HuR antisense overexpression plasmid were treated with 20 ng/ml IFN-γ (I) and 500 ng/ml DON (D) for 2 h. Cellular lysates were subjected to Western blotting. All results are representative of two or three independent experiments.

**FIGURE 8.** Effects of ribosomal insults on HuR translocation and its binding to the SOCS3 transcript. (A) HCT-8 cells were treated with vehicle or 20 ng/ml IFN-γ in the presence or absence of ribosomal stress agents (500 ng/ml DON or 50 ng/ml ANS) for 1 h. Cells were then stained and observed under a confocal microscope (original magnification ×1800). (B) The relative amount of cytosolic HuR protein/total HuR was determined by calculating (cytosolic density of HuR outside of DAPI-stained area in nine cells)/(total density of HuR in nine cells). Values were collected from five fields, and each value was divided by the average value of the control group. Different letters over bars with the SD represent significant differences between groups, as determined by unpaired matched comparisons (p < 0.05). (C) RNA immunoprecipitation assays were performed to measure HuR protein bound to the SOCS3 transcript in cells. Cells were treated with 20 ng/ml IFN-γ (I) in the presence or absence of 500 ng/ml DON (D) for 1 h. Immunoprecipitated transcript was measured using real-time RT-PCR. I, D, and A represent IFN-γ, DON, and ANS, respectively. All results are representative of two or three independent experiments. *p < 0.05 versus control group.
BAFF is a vital homeostatic cytokine for B cell development and is particularly important in the context of IgA class switching (26, 27). Mucosal epithelial cells located in crypt pockets potentiate their actions via BAFF on naive B cells expressing activation-induced cytidine deaminase, an enzyme required for IgA class switching. Mucosal epithelial cells have the potential to initiate frontline Ig class switching through locally abundant BAFF and IL-10 after sensing microbial products via TLRs. In addition to the direct action of epithelial BAFF and IL-10 on class switching in naive B cells, mucosal epithelial cells trigger dendritic cell maturation to produce BAFF via TSLP, which amplifies local BAFF concentrations for B cell development. However, the hyperproduction of BAFF alone is not sufficient to induce IgA-linked nephritis. Accordingly, more profound factors in the mucosal niche must also be responsible for mucosa-associated renal pathogenesis. Commensal bacteria in mucosa are one such potent factor (e.g., BAFF overproduction causes commensal microflora–dependent IgA nephritis in animal models) (67). Moreover, the frontline epithelial switching in a T cell–independent manner (16). This alternative switching of IgA could be due to the sufficient levels of BAFF produced locally in the intestine, although the levels of circulating BAFF are too low to trigger differentiation of B lymphoid cells.

In terms of signaling intervention, ribosomal insult was found to attenuate IFN-γ–activated JAK/STAT signal transduction and subsequent BAFF induction in gut epithelial cells. Mechanistically, ribosomal insults led to the superinduction of SOCS3 by enhancing its mRNA stability via HuR to SOCS3 transcript binding. Furthermore, upregulated SOCS3 contributed to the blocking of JAK/STAT-linked signal, which mediated BAFF suppression by ribosomal stress. These findings show that ribosomal stress–induced SOCS3 plays a regulatory role in epithelial BAFF production. In addition to BAFF regulation, SOCS3 could regulate epithelial proliferation in response to mucosal injuries. In a previous study (68) in ulcerative colitis and in vitro models, SOCS3 overexpression limited injury–induced epithelial hyperproliferation and inflammation–associated colon cancer by regulating both STAT and NF-κB signals. Thus, in terms of wound healing, ribosomal insult–induced SOCS3 could retard the regeneration or restitution process after ribosomal stress–induced mucosal injuries. Moreover, the growth–inhibitory action via SOCS is consistent with the findings of recent studies on the suppression of growth–related signals (69, 70). According to these reports, DON (a chemical ribosomal stress agent) induced SOCS proteins after the onset of proinflammatory cytokine decline, and this induction was involved in impaired growth hormone signaling in liver. Moreover, increasing evidence indicates mucosal ribosomal stress disrupts epithelial integrity (38, 39, 59), particularly regulating cellular growth and survival (71, 72). Furthermore, some ribosomal insults can induce G2/M cell cycle arrest via p21 expression in human epithelium (73). Because SOCS3 also can regulate cellular proliferation, assessment of the involvement of SOCS3 in epithelial cell arrest by ribosomal stress was warranted. In the current study, ribosomal insults superinduced SOCS3 expression in the presence of IFN-γ, which contributed to the reduction in IFN-γ–induced BAFF expression. Furthermore, SOCS3 directly interrupted receptor–associated adaptor JAK2, which restrains JAK/STAT-signaling cascades. Mechanistically, the superinduction was due to the stabilization of SOCS3 mRNA by HuR, which was exported from cytosol and bound to the SOCS3 transcript in response to ribosomal insults. Despite the contribution made by SOCS3 to BAFF suppression, we cannot exclude the possibility that translational inhibition by mucosal ribosomal stress is responsible, because most ribosomal insults lead to global arrest of protein synthesis by interfering with ribosomal function.
production of BAFF is quite dependent on the epithelial recognition of luminal commensal microorganisms through TLRs, such as TLR3, for viral dsRNA (74). Constitutive triggering by commensal-sensitive factors in the mucosal niche results in excess mucosal commensal–reactive IgA production to bleach mucosal-peripheral compartmentalization, which would be important for IgA-associated pathogenesis. In addition to the direct effects of proinflammatory IFN-γ, commensal bacteria–derived components are supposed to affect epithelial BAFF production. However, in our cell culture system, the production of BAFF by bacterial endotoxins was marginal compared with the production induced by IFN-γ (data not shown). This finding is also consistent with a previous study, in which bacterial endotoxins were not found to trigger BAFF expression in lung epithelial cells (25). However, more sophisticated in vivo examinations are required to address commensal-associated factors triggering epithelial BAFF production and its regulation by SOCS3 proteins. SOCS proteins have been considered key pathophysiological regulators of immune homeostasis and autoinflammatory diseases. In particular, SOCS1 and SOCS3 are strong inhibitors of JAK because they contain kinase-inhibitory regions at their N termini. The present study suggests that SOCS3 acts as a novel regulator of epithelial BAFF gene expression. In terms of molecular intervention, epithelial SOCS3 may be a promising therapeutic target for the treatment of BAFF-associated mucosal diseases. Furthermore, epithelial ribosomal dysfunction could be a potent point for regulating BAFF-linked immune responses and human diseases.

Disclosures
The authors have no financial conflicts of interest.

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