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Roseburia intestinalis-derived flagellin is a negative regulator of intestinal inflammation

Yongsheng Quan [a, b], Kerui Song [c], Yan Zhang [d], Changxin Zhu [a, b], Zhaohua Shen [a, b], Shuai Wu [a, b], Weiwei Luo [a, b], Bei Tan [a, b], Zhenyu Yang [a], Xiaoyan Wang [a, b, *]

[a] Department of Gastroenterology, The Third Xiangya Hospital of Central South University, Changsha, Hunan, China
[b] Hunan Key Laboratory of Nonresolving Inflammation and Cancer, Changsha, Hunan, China
[c] School of Materials Science and Engineering, Central South University, Changsha, Hunan, China
[d] Department of Ultrasound, The Third Xiangya Hospital of Central South University, Changsha, Hunan, China

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ABSTRACT

Our previous study showed that the *Roseburia intestinalis* (*R. intestinalis*), one of the dominant intestinal bacterial microbiota, was significantly decreased in Crohn's disease patients and protected colon epithelial cells from inflammatory damage. However, the roles of lncRNAs in *R. intestinalis* flagellin-mediated anti-inflammation remain unclear. In this study, we investigate global lncRNA expression profiles using microarray analysis of ulcerative colitis samples from DSS/Flagellin-challenged mice and identified a Flagellin-induced upregulated lncRNA (HIF1A-AS2). Flagellin induced HIF1A-AS2 expression in a dose- and time-dependent manner via p38-stat1 activation. Selective pharmacological inhibitors of Stat1 and p38, and genetic knockdown of these genes abolished Flagellin-induced HIF1A-AS2 expression. In addition, luciferase reporter assay showed that Flagellin activated HIF1A-AS2 promotor via increasing stat1 phosphorylation. Silencing of HIF1A-AS2 abolished Flagellin-mediated anti-inflammatory effects, evaluating by upregulation of cytokines expression, including TNF-α, IL-1β, IL-6 and IL-12, but not TNFβ. In addition, knockdown of HIF1A-AS2 significantly increased p65 and Jnk phosphorylation, and sufficiently abolished Flagellin-mediated anti-inflammatory affects in vivo. Our study provides new insights into the mechanisms that lncRNAs regulate flagellin-mediated alleviation of colonic inflammation. It is indicated that HIF1A-AS2 may be a modulator of intestinal inflammation and represent a novel target for future therapeutics.

1. Introduction

Ulcerative colitis (UC) is a chronic inflammatory bowel disease (IBD) [1]. The pathogenesis of UC involves in a complex interplay between abnormal gut microbiota, immune response dysregulation, environmental changes, and gene variants [2]. Although many investigations have tried to illustrate novel pathogenic factors associated with UC, a full understanding of UC pathogenesis is unclear [3]. Effective medical treatments exist for these chronic conditions, but UC treatment is far from optimal, and patient outcomes can be unsatisfactory.

The microbiota plays critical roles in immune system. The microbiota contains over 15,000 distinct species and more than 2×10^6 distinct genes. We previously found that the species *R. intestinalis* was significantly decreased in Crohn's disease patients and induced anti-inflammatory responses by increasing Treg cell numbers and inhibiting IL-17 secretion [4,5], indicating that this species is closely related to the development of IBD. Flagellin is the primary structural component of bacterial flagella, which plays a major role in inflammatory bowel disease. Extracellular flagellin is recognized through toll-like receptor 5 (TLR5), whereas intracellular flagellin is recognized through a pathway apparently involving both interleukin-converting enzyme protease-activating factor [6]. Flagellin activates its ligation, Toll-like receptor (TLR) 5, and then Toll-like receptors trigger a signaling cascade that leads to NF-κB translocation from the cytoplasm to the nucleus to control transcriptional regulation [7]. Flagellin promotes migration of intestinal epithelial cells through activation of the p38 MAPK

* Corresponding author. Department of Gastroenterology, The Third Xiangya Hospital of Central South University, Tongzipo Road 138, Changsha, Hunan, 410013, China.
E-mail address: xiaoyanwangxy3@163.com (X. Wang).

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pathway [8]. Flagellin treatment also stimulated stat1 activation [9]. Through these pathways, flagellin can activate many genes, including those with direct antibacterial function as well as anti-apoptotic function in epithelial cells, which allows cells to stay alive in response to challenges [10]. The role of *R. intestinalis*-derived flagellin in inflammatory bowel disease remain unclear.

Long non-coding RNAs (lncRNA), a class of nonprotein-coding transcripts with greater than 200 bases in length, are involved in many physiological and pathological processes. Previous studies indicated important role of lncRNA on regulation of inflammatory bowel disease-related inflammatory responses [11,12]. However, the roles of lncRNAs in *R. intestinalis* flagellin-related inflammation remain unclear.

In this study, we investigate global lncRNA expression profiles using microarray analysis of ulcerative colitis samples from DSS/Flagellin-challenged mice and propose a model whereby stat1 signaling induces the upregulation of lncRNA-HIF1A-AS2, which serves as a negative regulator of inflammatory responses.

2. Material and methods

2.1. LncRNA microarray analysis

Total RNA was extracted from nine colonal samples (three control mice, three DSS treated mice and three DSS plus Flagellin treated mice) by the RNeasy Mini Kit (Qiagen, GmBH, Hilden, Germany) according to the manufacturer's instructions. Purified total RNA from the same group was pooled in one and quantified using the NanoDrop 2000 spectrophotometer. The mixed total RNA was sent to Aksomics Co. Ltd. (Shanghai, China) to analyze lncRNA expression profiles using Mouse LncRNA Array V3.0 (8×60 K, including 35923 lncRNAs and 24881 coding genes). Array images were analyzed using GenePix 4000B, and Gene-Spring software was employed to finish the basic analysis of the raw data. Differentially expressed lncRNAs were identified through fold change. Volcano plot displayed the statistically significant differential expression of lncRNA with a threshold of fold change ≥ 2 and $q < 0.05$.

2.2. RNA extraction and qRT-PCR

Total RNA was extracted from cells or tissues with the use of TRIzol reagent (Invitrogen). RNA was reverse-transcribed using QuantiNova Reverse Transcription Kit (Cat no: 205413, Qiagen). Quantitative polymerase chain reaction (PCR) amplification was performed with an CFX96 Touch™ Deep Well Real-Time PCR Detection System (BioRad, Hercules, CA, USA) according to the manufacturer's instructions. Relative gene expression (HIF1A-AS2 and other inflammatory genes, normalized to endogenous control gene β-actin) was calculated using the comparative Ct method formula $2^{-\Delta\Delta Ct}$. The real-time PCR primer sequences are shown in Table 1.

Table 1
The primer sequences used in qRT-PCR.

Gene	Primer sequences (5'->3')
HIF1A-AS2	Forward: AGGACCTAAGGCTCTGGCAC
	Reverse: GGGATGAGTGAAGCAGTTCTCA
TNFα	Forward: CACAGTGAAGTGCTGGCAAC
	Reverse: ACATTGGGTCCCCCAGGATA
IL-1β	Forward: ACCAAACCTCTTCGAGGCAC
	Reverse: ATCGTGCACATAAGCCTCGT
IL-6	Forward: TCTCAACCCCCAATAAATATAGGAC
	Reverse: GATGCCGTCGAGGATGTACC
IL-12	Forward: TTACCCTTGCACTTCTGAAGAGAT
	Reverse: GCCAGGCAACTCCCATTAGTT
TNFβ	Forward: GGGATTCCCAAGGGGTGACTC
	Reverse: TCAAAGGCAGGAAAGGCTGA
β-actin	Forward: GCCCTATAAAACCCAGCGGC
	Reverse: TCGATGGGGTACTTCAGGGT

2.3. Western blot

Protein was extracted from cells or tissues harvested at indicated times using radio immunoprecipitation assay (RIPA) lysis buffer supplemented with a proteinase inhibitor cocktail (Boster, Wuhan, China). Protein concentrations were measured using the BCA Protein assay kit (Thermo Scientific, Waltham, MA). Equal amounts of protein were separated by 10% SDS/PAG, followed by immunoblotting with the following primary antibodies: phospho-Stat1 (Tyr701) antibody (Rabbit monoclonal, diluted at 1:1000, 9167, Cell Signaling Technology), phospho-Stat1 (Ser727) antibody (Rabbit monoclonal, diluted at 1:1000, 8826, Cell Signaling Technology), Stat1 antibody (Rabbit monoclonal, diluted at 1:1000, 14994, Cell Signaling Technology), p38 MAPK antibody (Rabbit monoclonal, diluted at 1:1000, 8690, Cell Signaling Technology), NF-κB p65 antibody (Rabbit monoclonal, diluted at 1:1000, 8242, Cell Signaling Technology), Phospho-NF-κB p65 (Ser536) antibody (Rabbit monoclonal, diluted at 1:1000, 3033, Cell Signaling Technology), Jnk antibody (Rabbit polyclonal, diluted at 1:1000, 9252S, Cell Signaling Technology), JNK (phospho T183) antibody (Rabbit polyclonal, diluted at 1:1000, ab47337, Abcam), GAPDH antibody (Rabbit polyclonal, diluted at 1:1000, 5174, Cell Signaling Technology). Membranes were then incubated with peroxidase-conjugated secondary antibody, and specific bands were detected with a Bio-Rad (Hercules, CA) imaging system.

2.4. Luciferase reporter assay

For reporter assay, HIF1A-AS2 promoter was PCR amplified from mouse genomic DNA and cloned into the pGL3-Basic vector using the Fast-Fusion™ Cloning Kit (FFPC-C050, FulenGen, Guangzhou, China). Luciferase reporter constructs were co-transfected with an internal control plasmid, pRL-TK (Renilla luciferase reporter plasmid, Promega), into Caco-2 cells, followed by stimulation with Flagellin. The luciferase activity was determined with the Dual Luciferase Reporter Assay Kit (Promega) according to the manufacturer's instruction.

2.5. Generation of recombinant lentivirus

Lentivirus carrying the entire sequence of HIF1A-AS2 (Lv-HIF1A-AS2) was constructed with the Lenti-Pac HIV Expression Packaging Kit (GeneCopoeia, Guangzhou, China). Empty lentivirus was used as a negative control (Lv-EV). To generate lentivirus expressing shRNA against HIF1A-AS2 (Lv-sh-HIF1A-AS2), 3 siRNAs for mouse HIF1A-AS2 were designed and the one with the optimal knockdown efficiency was chosen to create shRNA and then recombined into lentiviral vectors. The target sequence is as follows: AAGCTGATCAAAGGGGCCTGGTC. The negative control lentivirus was designed to express nontargeting shRNA (Lv-shNC).

2.6. Cell culture and treatment

Caco-2 cells were obtained from ATCC and cultured in complete DMEM medium supplemented with 10% fetal bovine serum under 5% CO_2 and 37 °C conditions for further study. Selective pharmacological inhibitors (Bay-11-7082 (NF-κB), U0126 (extracellular signal-regulated kinase, Erk), SP600125 (c-Jun N-terminal kinase, Jnk), SB203580 (p38), and PF-04965842 (Janus kinase 1, Jak1)) were

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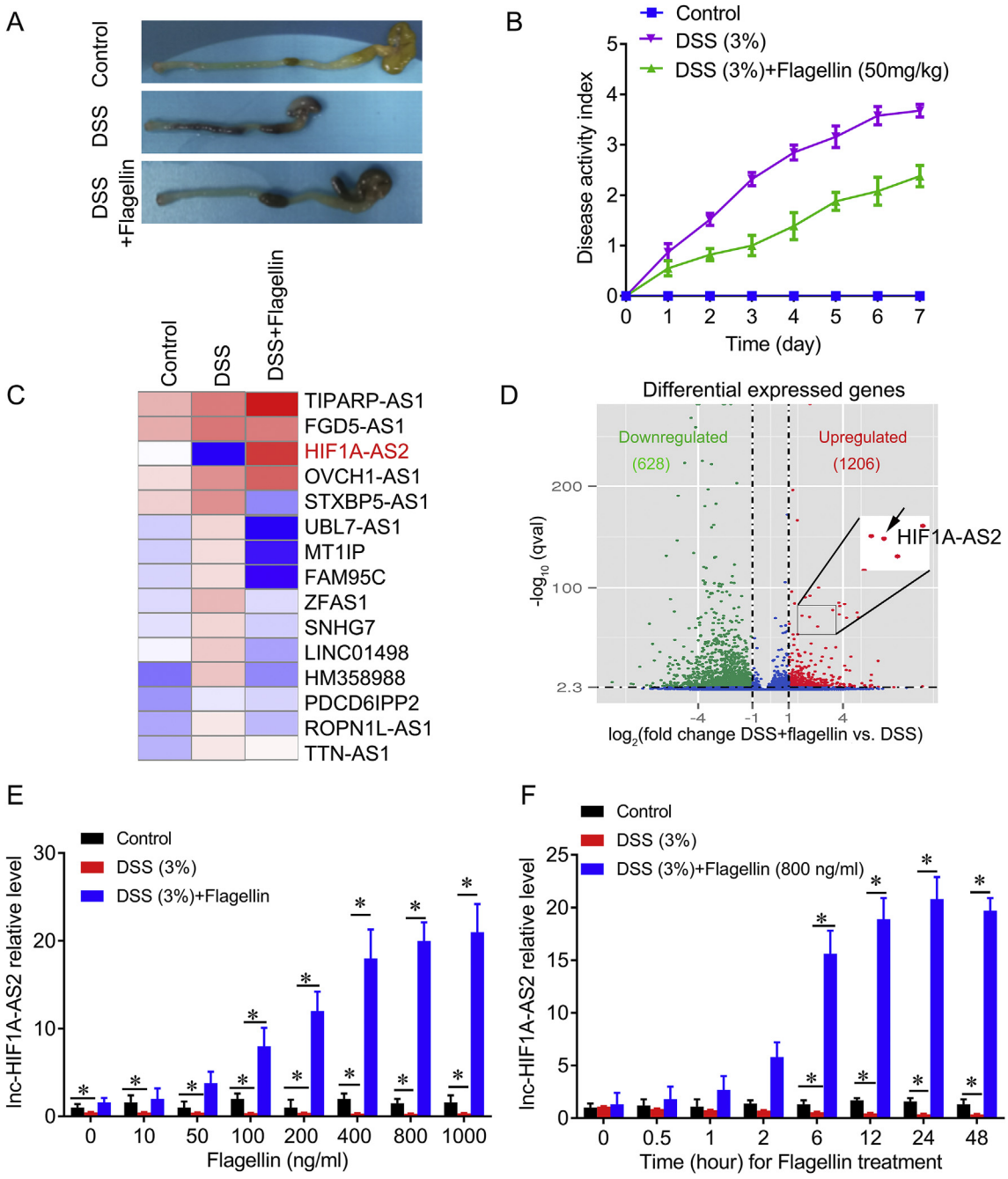

Fig. 1. Differentially expressed lncRNAs in colorectal endothelial cells treated with *R. intestinalis* flagellin. (A) The colorectum obtained from mice treated with DSS (3%), or along with *R. intestinalis* flagellin (50 mg/kg/day) for 7 days. (B) Disease activity index of the mice was evaluated every day. (C) The colorectal tissues were used to determine their lncRNA profiles using an lncRNA expression microarray. The cluster heatmap shows lncRNAs with expression change fold>2 from microarray data ($P < 0.01$). (D) A volcano plot showing the relationship between the q values and the magnitude of the differences in the expression values of the samples in the different groups. (E) The expression of lnc-HIF1A-AS2 in Caco-2 cells treated with DSS (3%) and Flagellin of concentration gradient for 24 h. (F) The expression of lnc-HIF1A-AS2 in Caco-2 cells treated with DSS (3%) and Flagellin (800 ng/ml) for different times. Data represent the mean ± SEM of three independent experiments. *$p < 0.05$. Two-tailed Student's *t*-test for two groups.

purchased from Selleck (Shanghai, China). Dextran sulfate sodium salt (DSS, cat no. 42867) were purchased from Merck Millipore and Sigma-Aldrich and 3% DSS were used to challenge cells and mice. *R. intestinalis* were cultured as our previously described [4]. Flagellin proteins from *R. intestinalis* were prepared as methods outlined previously [13].

Caco-2 cells were pretreated with these inhibitors for 2 h to assess the functional consequences of inhibition of these pathways on HIF1A-AS2 expression. Caco-2 cells were exposed to *R. intestinalis* flagellin proteins (800 ng/ml, prepared in DMEM with 10% fetal calf serum and antibiotics) for various hours (0, 0.5, 1, 2, 6, 12, 24 or 48) or a series of concentrations (0, 10, 50, 100, 200, 400, 800 or 1000 ng/ml) for 24 h.

2.7. Animal experiments

All the animal experimental protocols were approved by the Ethics Committee of Central South University, China, and were

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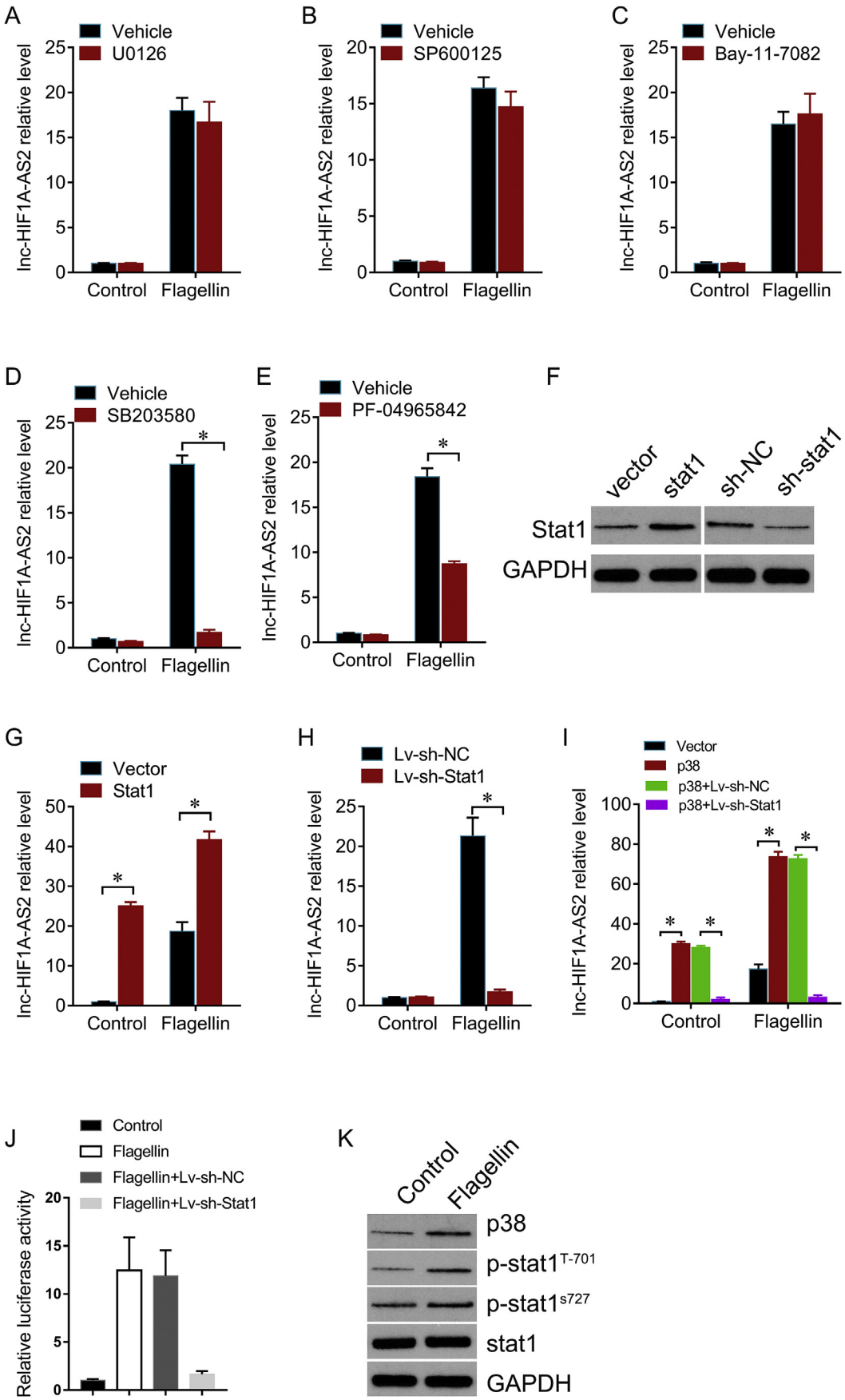

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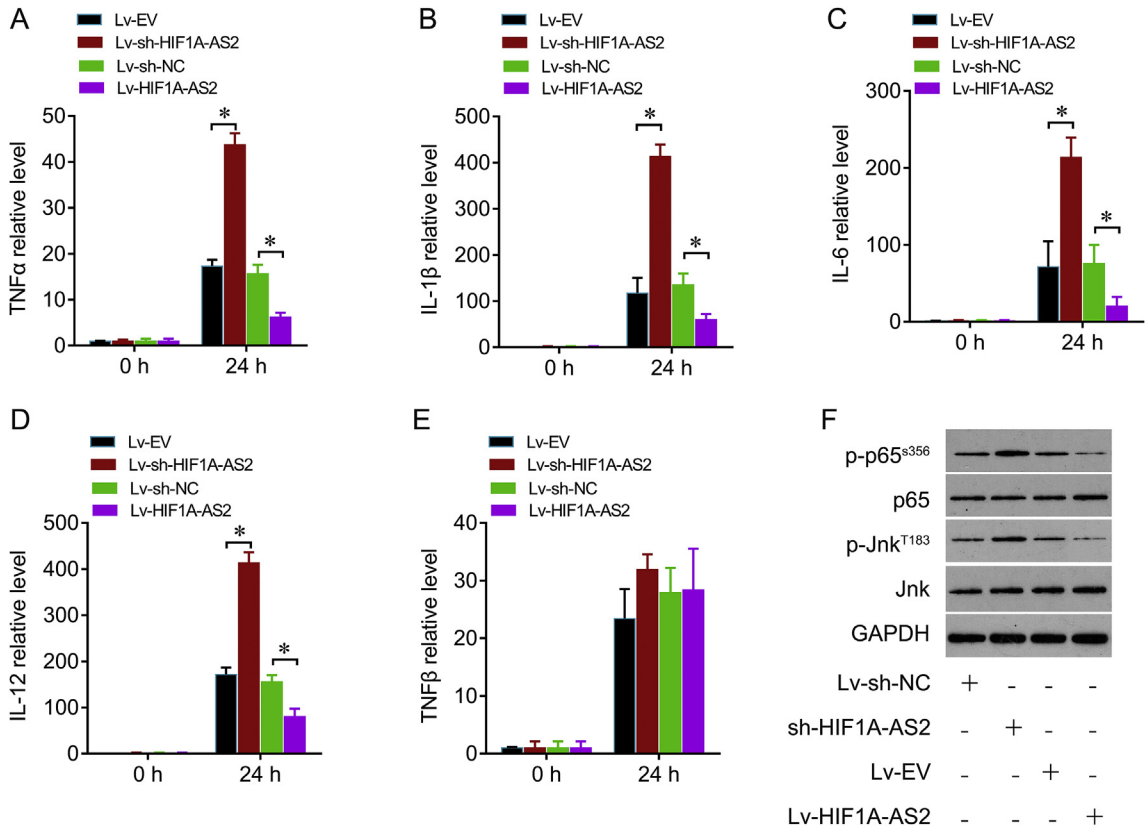

Fig. 3. Knockdown of HIF1A-AS2 promotes inflammatory responses in Caco-2 cells. (A–E) Effects of HIF1A-AS2 overexpression or HIF1A-AS2 knockdown on the expression of inflammatory factors in Caco-2 cells, as determined by qRT–PCR. Data represent the mean ± SEM of three independent experiments. *P < 0.05 vs. Lv-EV or Lv-shNC group. (F) Effects of HIF1A-AS2 on the activation of NF-κB pathways in Caco-2 cells, as determined by Western blot. Data represent the mean ± SEM of three independent experiments. *p < 0.05. Two-tailed Student's t-test for two groups.

conducted in accordance with the National Institutes of Health (NIH) Guide for the Care and Use of Laboratory Animals. Male C57BL/6 mice (8–12 weeks) were purchased from the Slac Animal Laboratory (Changsha, China). All the mice were fed standard food and water ad libitum and kept in a constant environment, 20 ± 2 °C and 12 h light/dark cycle. Experimental colitis was induced by the p.o. administration of 3% DSS for 7 days. The mice were divided into three groups: normal control group (healthy mice given only water to drink, n = 6), DSS model group (mice administered DSS in their drinking water, n = 6) and DSS plus Flagellin group (DSS mice also administered Flagellin p.o. (50 mg/kg), n = 6). The disease activity index (DAI) scores were assessed daily for the severity of colitis as previously described [4]. Lentivirus (5×10^9 pfu/mouse) were delivered into mice by tail veil injection, 3 days before model established. At day 8, the mice were killed via injecting over dose pentobarbital sodium, and the colon were harvested for subsequent use in various assays.

2.8. Histological analysis

Once removed, the colons were immediately fixed in 10% buffered formalin. Sections were stained with H&E for evaluating morphology and with periodic-Schiff (PAS) reagent for measuring goblet cell density. The number of goblet cells was measured in 3 sections from each colon, using image-analysis software (NIS Elements Software, version 3.0, BR, Nikon) and expressed as number of goblet cells per villus.

2.9. Transmission electron microscopy (TEM)

TEM was used to evaluate the effect of Flagellin on the intestinal mucosal barrier at the ultrastructural level. Intestinal mucosal fragments were fixed with 2.5% glutaraldehyde solution overnight at 4 °C and with 1% osmic acid for 2 h followed by 3-time washes with PBS. After the specimens were embedded in an Epon/Araldite mixture and stained with uranyl acetate and lead citrate, the intestinal mucosal barrier was observed under a 1230 type transmission electron microscope (Electron Co, Tokyo, Japan) and photographed.

Fig. 2. *R. intestinalis* flagellin upregulates HIF1A-AS2 in Caco-2 cells via p38-Stat1 signaling. (A–E) Caco-2 cells were pretreated with selective pharmacological inhibitors U0126 (Erk, 50 μM), SP600125 (Jnk, 50 μM), Bay-11-7082 (NF-κB, 10 μM), SB203580 (p38, 50 μM) or PF-04965842 (Jak1, 50 nM), then cells were activated by Flagellin (800 ng/mL, 24 h). Expression of HIF1A-AS2 was detected by qRT-PCR. (F) Western blot analysis for Stat1 after overexpression or knockdown on Caco-2 cells. (G–H) Effects of Stat1 overexpression on the expression of HIF1A-AS2 in Caco-2 cells. (H) Effects of Stat1 knockdown on the expression of HIF1A-AS2 in Caco-2 cells. (I) Effects of p38 overexpression on the expression of HIF1A-AS2 in Caco-2 cells pretreated with sh-NC or sh-Stat1. (J) Luciferase reporter constructs containing HIF1A-AS2 promoter were co-transfected with an internal control plasmid pRL-TK, and with sh-NC or sh-Stat1 into Caco-2 cells, followed by Flagellin challenge (800 ng/mL, 24 h). The relative luciferase activities are expressed as a percent of values determined in control group. (K) The phosphorylation of Stat1 induced by Flagellin. Data represent the mean ± SEM of three independent experiments. *p < 0.05. Two-tailed Student's t-test for two groups.

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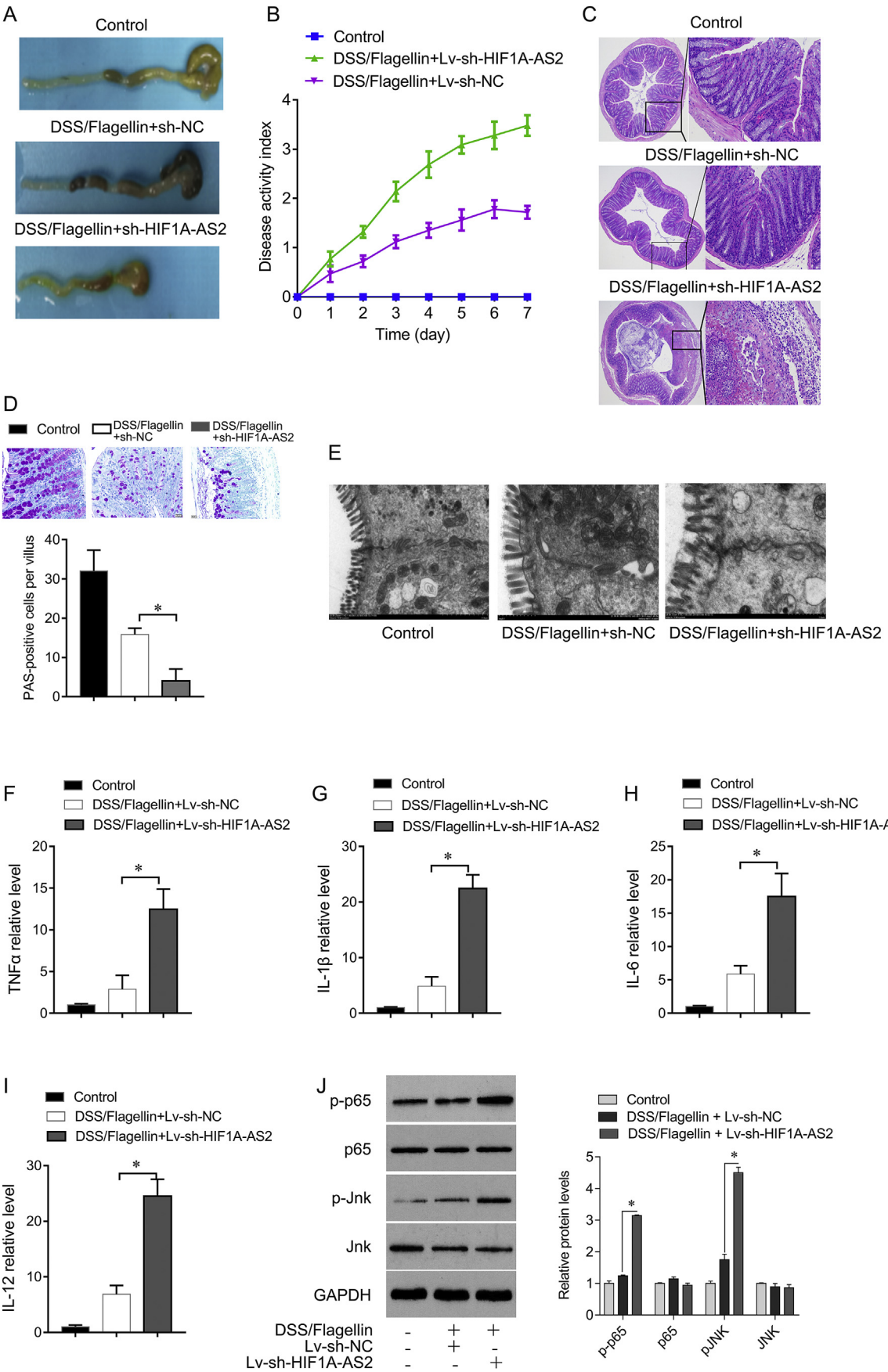

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2.10. Statistical analysis

GraphPad Prism software (GraphPad Software Inc., La Jolla, CA) was used for statistical analyses. Values are expressed as means ± SEM of at least three independent experiments. Student's *t*-test was used to assess the statistical significance of the differences between two groups. For multiple groups, significance was evaluated by one-way ANOVA with Bonferroni test. $P < 0.05$ was considered statistically significant.

3. Results

3.1. Differentially expressed lncRNAs in flagellin-treated colonal endothelial cells

We established DSS combined with Flagellin-induced ulcerative colitis in C57BL/6 mice. The mice treated with DSS has shorter colon and higher disease activity index than the control mice, whereas the mice simultaneously treated with DSS and Flagellin has longer colon and lower disease activity index than the mice treated with DSS alone (Fig. 1A and B). To identify the lncRNAs that are involved in Flagellin alleviated ulcerative colitis, we performed a microarray analysis in colonal endothelial cells obtained from C57BL/6 mice challenged by DSS and Flagellin. Flagellin, which is a toll-like receptor 5 (TLR5) ligand, induced numerous differentially expressed lncRNAs. In the volcano plot, 1834 lncRNAs were represented, of which, 1206 were significantly upregulated (red plots) and 628 were downregulated (green plots) when filtered with a threshold of a fold change ≥ 2 and $q < 0.05$ (Fig. 1C and D). LncRNA-HIF1A-AS2 was among the most highly induced upregulated lncRNAs and was abundantly expressed in colonal endothelial cells. The response of lncRNA-HIF1A-AS2 to TLR5 signaling was confirmed by qRT-PCR. HIF1A-AS2 expression in Caco-2 cells was induced by Flagellin in a time- and dose-dependent manner, which peaked at 24 h at a concentration of 0.8 μg/mL (Fig. 1E and F).

3.2. Upstream mediators of HIF1A-AS2 expression in colonal endothelial cells

Flagellin acts through distinct pathways leading to endothelial cells activation. We used selective pharmacological inhibitors, including NF-κB inhibitor Bay-11-7082, extracellular signal-regulated kinase (Erk) inhibitor U0126, c-Jun N-terminal kinase (Jnk) inhibitor SP600125, p38 inhibitor SB203580, and Janus kinase 1 (Jak1) inhibitor PF-04965842 to assess the functional consequences of inhibition of these pathways on HIF1A-AS2 expression. Pretreatment with SB203580 completely abrogated Flagellin-induced HIF1A-AS2 upregulation in Caco-2 cells. In addition, the Jak1 inhibitor PF-04965842 exerted partial inhibitory effects on Flagellin-induced HIF1A-AS2 expression (Fig. 2, A-E).

The overexpression and knockdown efficiency for the Stat1 and Stat1-shRNA was shown by Western blot (Fig. 2 F). Overexpression of Stat1 significantly enhanced Flagellin-induced HIF1A-AS2 expression in Caco-2 cells, whereas silencing of Stat1 completely abrogated Flagellin-induced HIF1A-AS2 expression (Fig. 2 G-H). Our previous studies demonstrated that inhibition of the p38 pathway by SB203580 induced a reduction in HIF1A-AS2 expression in response to Flagellin. Consistently, enforcing p38 expression increased the HIF1A-AS2 levels in Caco-2 cells either under resting conditions or after activation by Flagellin, whereas these effects could be completely abrogated by knockdown of Stat1 (Fig. 2 I). To further explore the mechanisms underlying the HIF1A-AS2 elevation in Flagellin-activated Caco-2 cells, the HIF1A-AS2 promoter-luciferase reporter construct was established and transfected into Caco-2 cells. Flagellin treatment significantly increased the luciferase activity, whereas this increment was abrogated by knockdown of Stat1 (Fig. 2 J). In addition, our studies showed that Flagellin treatment led to phosphorylation of Stat1 at serine 727 (S-727) and tyrosine 701 (T-701) (Fig. 2 K). These results demonstrated that the Flagellin-p38-Stat1 pathway was responsible for the Flagellin-induced upregulation of HIF1A-AS2 in colonal endothelial cells.

3.3. HIF1A-AS2 inhibits upregulation of inflammatory genes through suppressing NF-κB signaling pathway activation

To define the functional role of HIF1A-AS2 in Flagellin-mediated anti-inflammatory response in colonal endothelial cells, HIF1A-AS2 was enforced or silenced by lentivirus-mediated gene expression. Enforcing or silencing HIF1A-AS2 expression did not alter the basal levels of inflammatory factors, including TNFα, IL-1β, IL-6, IL-12 and TNFβ. However, in Flagellin-activated Caco-2 cells, ectopic HIF1A-AS2 expression inhibited the activation of all of these inflammatory factors, and HIF1A-AS2 silencing led to increase in TNFα, IL-1β, IL-6, and IL-12 expression (Fig. 3A–E). We further examined the correlation between HIF1A-AS2 and the phosphorylation of critical signaling proteins of NF-κB pathway. The phosphorylation levels of p65 and Jnk in Caco-2 cells were dramatically downregulated after HIF1A-AS2 overexpression; silencing HIF1A-AS2 increased the phosphorylation levels of p65 and Jnk (Fig. 3F). These data suggest that HIF1A-AS2 acts as a negative regulator to NF-κB signaling pathway activation, thereby downregulating inflammatory genes in Caco-2 cells.

3.4. HIF1A-AS2 knockdown protects aggravates ulcerative colitis

We investigated the effects of HIF1A-AS2 in mice with ulcerative colitis induced by DSS/Flagellin challenge. sh-HIF1A-AS2 recombinant lentivirus were administrated via the tail vein. Lv-sh-HIF1A-AS2 administration showed remarkable aggravation in mice with shorter colon and higher disease activity index than Lv-sh-NC administrated mice (Fig. 4A, B). An examination of the pathology of the colon tissue showed that the DSS/Flagellin -treated mice developed increased colon inflammation, and goblet cell losing, whereas more colon lesions were observed in the mice receiving Lv-sh-HIF1A-AS2, with a higher disease activity index, less goblet cell survival and increased inflammatory cell infiltration as demonstrated by H&E and PAS staining (Fig. 4B and C, D). The intestinal mucosal barrier in the control mice showed integrity mediated by colonic epithelial cells (CECs), whereas CECs in the DSS/Flagellin-treated mice displayed significant edema. Moreover, the DSS/Flagellin-treated mice showed a loss of intestinal mucosal barrier integrity, with pericellular edema and vacuolation and membrane damage. However, administration of Lv-sh-HIF1A-AS2 aggravate the injury mentioned above (Fig. 4E). Moreover, silencing HIF1A-AS2 resulted in a significant upregulation in gene

Fig. 4. Knockdown of HIF1A-AS2 aggravates ulcerative colitis. (A) The colorectum obtained from mice treated with DSS (3%) along with Flagellin (50 mg/kg/day) or combined with Lv-sh-NC or Lv-sh-HIF1A-AS2 for 7 days. (B) Disease activity index of the mice was evaluated every day. (C) H&E staining was performed to evaluate the mucosal damage. (D) periodic acid Schiff (PAS) staining was performed to evaluate the goblet cells. (E) Ultrastructural morphology of the intestinal mucosal barrier after indicated treatment. (F–H) Effects of HIF1A-AS2 knockdown on the expression of inflammatory factors in colonal tissues, as determined by qRT–PCR. (J) Effects of HIF1A-AS2 knockdown on the activation of NF-κB pathways in colonal tissues, as determined by Western blot. Data represent the mean ± SEM of three independent experiments. *$p < 0.05$. Two-tailed Student's t-test for two groups.

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expression of the pro-inflammatory cytokines TNFα, IL-1β, IL-6, and IL-12 (Fig. 4F–I). Western blot assays showed that Lv-sh-HIF1A-AS2 pretreatment significantly reduced p65 and Jnk phosphorylation in the colon tissues compared with in the Lv-sh-NC treated group (Fig. 4 J). Together, these results showed that HIF1A-AS2 knockdown could aggravate DSS-induced ulcerative colitis.

4. Discussion

Flagellin has been involved in many inflammatory diseases by activating immune response. Few studies investigate the role of lncRNAs on flagellin-mediated anti-inflammation effects. In this study, we investigated lncRNA expression profiles in response to ulcerative colitis induced by DSS and identified a *R. intestinalis* flagellin-induced upregulated lncRNA (HIF1A-AS2). Activation of the flagellin-p38-Stat1 pathway transcriptionally promoted lncRNA HIF1A-AS2 expression. *R. intestinalis* flagellin-induced HIF1A-AS2 effectively relieved the inflammatory responses in vitro and in vivo.

Cells in the intestinal mucosa (both epithelial and immune cells) are quite responsive to flagellin [14]. Flagellin as a TLR agonists stimulate functional activation and cytokine gene expression via the extracellular signal regulated kinase 1/2 (ERK1/2) MAP kinase and IkB kinase cascade by inducing the phosphorylation of Rap1, which culminates in the activation of the canonical nuclear factor-kB pathway [15]. Epithelial cells and macrophages use markedly different signaling pathways to respond to flagellin. Epithelial cells activate a panel of host defense genes on sensing basolateral flagellin through TLR5. Flagellin stimulates cytoprotectants and growth factors secretion through an ERK1/2-dependent activation of NF-κB [16]. Flagellin also activates the MAPKs p38, c-jun N-terminal kinase (JNK), inducing IL-8 production in intestinal epithelial cells [17]. In addition, the region of the flagellin molecule recognized by macrophage and epithelial cells is highly variable even among closely related bacteria [18]. The present study showed that *R. intestinalis* flagellin induced HIF1A-AS2 expression in a dose- and time-dependent manner via p38-stat1 activation. Selective pharmacological inhibitors of Stat1 and p38, and genetic knockdown of these genes abolished flagellin-induced HIF1A-AS2 expression. In addition, luciferase reporter assay showed that *R. intestinalis* flagellin activated HIF1A-AS2 promotor via increasing stat1 phosphorylation. Stat1 mediates the transduction of cell transmembrane signal into the nucleus and directly regulates the transcription of the target gene [19]. Under the stimulation of the external signal, Stat1 can be activated through the phosphorylation of Tyr701 and Ser727 to regulate its target genes by combining with its specific binding element [20]. Using mass spectrometry analysis, Hu YW et al. revealed that Stat1 interacted with lncRNA PLAC2 promoter, but the cytoplasmic lncRNA PLAC2 inhibited Stat1 nuclear transfer, thereby decreasing ribosomal protein 36 expression, inhibiting cell proliferation and inducing cell cycle arrest in glioma cells [21]. Thus, it was reasonable that *R. intestinalis* flagellin mediated stat1 nuclear translocation, thereby activating promotor of HIF1A-AS2.

We further investigated the biology function of HIF1A-AS2. Silencing of HIF1A-AS2 abolished *R. intestinalis* flagellin-mediated anti-inflammatory effects by increasing cytokines expression, including TNF-α, IL-1β, IL-6 and IL-12, but not TNFβ. In addition, we explored the downstream pathway whereby HIF1A-AS2 carries out its functions. Knockdown of HIF1A-AS2 significantly activated *R. intestinalis* flagellin-inhibited p65 and Jnk phosphorylation. Flagellin increased binding of p65 to human beta-defensin-2 gene promoter sequences to induce its expression in Caco-2 cells [22]. Flagellin also delays neutrophil apoptosis through activation of ERK1/2 MAPK and NF-κB during infections with flagellated bacteria [23]. Flagellin activates NF-κB through MyD88, TRIF (a Toll-interleukin 1 receptor (TIR) domain-containing adaptor) or TLR5, and finally controls cytokines release [24,25]. We here find a novel *R. intestinalis* flagellin effector lncRNA-HIF1A-AS2. Overexpression of HIF1A-AS2 enhanced *R. intestinalis* flagellin-mediated anti-inflammation effects, whereas HIF1A-AS2 knockdown sufficiently abolished these effects in vitro and in vivo.

In summary, our study provides new insights into the mechanisms that lncRNAs regulate *R. intestinalis* flagellin-mediated alleviation of colonic inflammation. HIF1A-AS2, which is a *R. intestinalis* flagellin-induced lncRNA in gut epithelium, inactivates NF-κB/Jnk pathway and thus inhibits inflammatory responses. Our study indicates that HIF1A-AS2 may be a modulator of intestinal inflammation and represent a novel target for future therapeutics.

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Transparency document

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Conflicts of interest

None.

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