LncRNA-BG Inhibited Th17 Cells Differentiation By Targeting RORγt Protein.

hanlin he
Central South University
xiangjie qiu
Central South University
mingming qi
Central South University
Ousman Bajinka
Central South University
ling qin
Xiangya Hospital Central South University
YURONG TAN (yurongtan@csu.edu.cn)
Central South University https://orcid.org/0000-0002-0283-2105

Research

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Abstract

Background: In our previous study, we obtained lncRNA-BG related to COPD through high-throughput screening, but we could not determine the specific mechanism involved. To this responds, here, we designed this study to verify whether lncRNA-BG could regulate the differentiation of Th17 cells and its mechanism.

Methods: The interaction between lncRNA-BG and RORγt protein was predicted using bioinformatics approaches. This was then confirmed by RNA pull down and dual luciferase reporter assay. The correlation between lncRNA-BG and Th17 cell differentiation was verified among patients with COPD and *in vitro* culture experiment. Meanwhile, the regulatory effect of lncRNA-BG on Th17 cell differentiation was determined by regulation the expression level of lncRNA-BG.

Results: LncRNA-BG could bind with RORγt protein and inhibit the differentiation of Th17 cells. LncRNA-BG was significantly negatively correlated with Th17 differentiation in patients with COPD and *in vitro* experiment. The decrease level of LncRNA-BG could promote Th17 differentiation, while the increase level of LncRNA-BG could inhibit Th17 differentiation.

Conclusion: LncRNA-BG directly targets RORγt protein, inhibits the mutual binding of RORγt and IL-17 gene promoter, and eventually inhibits Th17 differentiation. LncRNA-BG as a potential target may confer applications in the clinical treatment and diagnosis of Th17-related diseases.

Background

The differentiation disorder of Th17 cell is studied with a growing evidence to be associated with many autoimmune diseases such as multiple sclerosis (MS), rheumatoid arthritis (RA), psoriasis, Crohn's disease (CD), obesity and metabolic syndromes[1-3]. An increased expression of IL-17A signal in chronic obstructive pulmonary disease (COPD) is increasing activating neutrophils and macrophages, and this leads to a high degree of airway obstruction in COPD patients[4,5]. After the stimulation of T cell receptors, the initial $CD4^+$ T cells are induced to differentiate into Th17 cells by TGF-β, IL-6, IL-23, IL-21, IL-1β and STAT3, producing the signature cytokines IL-17A, IL-17F, IL-21 and IL-22[6-8]. These cytokines activate many types of inflammatory cells such as epithelial cells, endothelial cells and fibroblasts to secrete IL-6, IL-8, G-CSF, ICAM-1 and so on. They can activate more inflammatory cells, mediators and cytokines to promote inflammatory response through MAP kinase pathway and NF-kappaB pathway[9-11]. Moreover, IL-17A can mobilize, recruit and activate inflammatory cells especially neutrophils to release inflammatory cytokines, thereby inducing the secretion of mucus and ultimately enhancing airway hyperresponsiveness (AHR)[12-14]. In a mouse model of asthma, the deficiency of IL-17A receptor reduced the recruitment of antigen-induced neutrophils and eosinophils, and also reduced the airway inflammatory response. Other studies also reported that the AHR were significantly inhibited in the mouse model of IL-17A gene knockout[15,16].

The mechanism associated with Th17 cell development and differentiation is extremely complex. RORγt is a subtype of RORγ, which is a member of the orphan nuclear receptor family of retinoic acid, expressed on immune cells. It is the major transcription factor in human and mouse that promote the differentiation of Th17 cells, but also regulate the expression and secretion of IL-17A[17,18]. T cells without RORγt were unable to differentiate into Th17 *in vitro*[19]. It has been found that digoxin could effectively delay and reduce the severity of autoimmune diseases in mice, such as multiple sclerosis and experimental autoimmune encephalomyelitis. They do this by changing the active conformation of RORγt, inhibiting the differentiation of Th17 cells, but couldn't affect the differentiation of other T cells[20]. This study suggested that targeting RORγt to inhibit the differentiation and function of Th17 cells is a promising therapeutics for Th17-mediated inflammation or autoimmune diseases.

LncRNAs are non-coding RNAs whose transcriptional lengths are more than 200 nucleotides. They confer little or may not involve in protein coding due to their lack of an open reading framework. LncRNAs can regulate various biological activities such as embryo development, cell proliferation, apoptosis, and differentiation in transcription, post-transcription, chromosome modification and other levels[21,22]. In our previous work, we obtained lncRNA-BG related to COPD through high-throughput screening, but the specific mechanism involved was unearthed. This present study was designed to confirm the regulatory role of lncRNA-BG in the differentiation of Th17 and provide scientific basis for new therapeutic target for Th17-related diseases.

Materials And Methods

Bioinformatics analysis

The sequence of LncRNA-BG was obtained from UCSC Genome Browser (http://genome.ucsc.edu/) and submitted to the CatRapid public database (http://s.tartaglialab.com/page/catrapid_group) and MEM Database https://biit.cs.ut.ee/mem/ to predict its interacting proteins.

RNA pull down Experiment

To verify the interaction between RORγt and lncRNA-BG, the RNA pull-down were carried out. LncRNA-BG was synthesized *in vitro* (Genscript, Nanjing, China) and biotin-labeled using Pierce^{TM} RNA3' End Desthiobiotinylation Kit. $CD4^+$ T cell extracts were incubated with Biotin labeled lncRNA-BG at 4 °C for 1 h. Then streptavidin magnetic beads (New England Biolabs, USA) were added and incubated at room temperature for 1 h. Lysate proteins in each reaction were detected by Western blot using mouse-anti human RORγt primary antibody (BD sciences, SD, USA) and goat-anti mouse secondary antibody (Boster, Wuhan, China).

Dual luciferase reporter assay

The IL-17 promoter was cloned into the luciferase expression reporter plasmid to construct pGL3-IL-17 (Genscript, Nanjing, China). LncRNA-BG and RORγt were cloned into pcDNA3.1 to construct pcDNA3.1-BG

and pcDNA3.1-RORγt (Genscript, Nanjing, China). After 293 cells were cultured in a 12-well plate for 24 hours (80% confluence), pGL3-IL-17,RORγt and lncRNA-BG were cotransfected into 293T cells in different combinations. The fluorescence signals were detected 48 h after transfection according to the manufacturer's instruction and relative fluorescence values (Luciferase / Renilla) were calculated.

Patients and health control

A total of 16 samples were recruited from Xiangya Hospital of Central South University, including 10 COPD patients and 6 healthy controls. These patients were diagnosed with COPD according to the criteria of the Respiratory Pathology Branch of the Chinese Medical Association and the Global Initiative for Chronic Obstructive Lung Disease (GOLD). All participants were provided written informed consent.

Collection and culture of $CD4^+$ T cells

The peripheral blood samples collected by the subjects were preserved in tubes with pretreatment of heparin sodium. Peripheral blood mononuclear cells (PBMCs) were isolated in Ficoll-Hypaque solution by density gradient centrifugation. $CD4^+$ T cells were then isolated using a positive selection magnetic isolation system (Miltenyi, Cologne, Germany) according to the manufacturer's instructions. The isolated $CD4^+$ T cells were assessed by flow cytometry. $CD4^+$ T cells with purity above 90% can be used for further analysis. The isolated $CD4^+$ T cells were routinely cultured in human T cell culture medium at 37 °C and 5% CO_2.

Human T-lymphocytic leukemia cell line Hut78 with mature T-cell induction-assisted characteristics was obtained from the American type culture collection cell library (ATCC). Hut 78 was cultured in IMDM medium (HyClone, Logan, USA) containing 20% fetal bovine serum and 1% penicillin-streptomycin at 37°C and 5% CO_2.

Th17 cell differentiation

Hut78 cells (2×10^6 Cells/mL) were cultured with 20 ng/mL of IL-6, 5 ng/mL of TGF-β, 25 ng/mL of IL-23, 5 μg/mL of anti-IFN-γ and 5 μg/mL of anti-IL-4 for 3 days under the activation of 2 μg/mL anti-CD3 and 5 μg/mL of anti-CD28. CD3, CD28, TGF-β and IL-23 were purchased from Sino Biological in Wuhan, China while Anti-IFN-γ and anti-IL-4 were purchased from Bioxcell in West Lebanon, USA .

Enzyme-linked immunosorbent assay (ELISA)

The concentrations of IL-17A in the supernatant of $CD4^+$ T cells of patients with COPD and TH17 cells cultured *in vitro* culture were measured by using ELISA according to the manufacturer's instructions (Fankew, Shanghai, China). In short, the supernatant was added to a 96-well plate with 100 μl per well. Appropriate biotin-binding antibodies (Fankew, Shanghai, China) were added to each well, and incubated at room temperature for 2 hours. After washing five times, substrate solution was added to each well and incubated in darkness at room temperature for 30 minutes. The optical density (OD) of each well was

detected at a wavelength of 450 nm. The concentration of IL-17A in the sample was calculated according to the standard curve.

Quantitative real-time PCR (qRT-PCR)

Total RNA in the cells was extracted with TRIzol reagent (TaKaRa, Beijing, China) according to the manufacturer's instructions. The concentration of the extracted RNA was detected by spectrophotometer. Through reverse transcription, RNA were detected using Reverse transcription kits (TaKaRa, Beijing, China) according to the procedure of product specification. The obtained cDNA was detected by qRT-PCR using SYBR®-Green and fluorescent quantitative PCR detection system (bimake, Houston, USA) according to the procedure of product specification. GAPDH was used as internal reference gene. The relative level changes of target genes were calculated by $2^{-\Delta Ct}$ method.

Cell transfection

To over express lncRNA-BG level in $CD4^+$ T cells, lentivirus vectors containing pcDNA-BG (GenePharma, Shanghai, China) were transfected into hut78 cells. 2 ml of hut78 cell suspension was inoculated into six-well culture plate for 24 hours before transfection.10 μl lentiviruses was added to each well and then 1μl of polybrene (GenePharma, Shanghai, China) was added to improve transfection efficiency for 24 hours. To down-regulate lncRNA-BG level in $CD4^+$ T cells, 10 μl of lentivirus vectors containing siRNA-BG (Jtsbio, Wuhan, China) and 1 μl of polybrene (Jtsbio, Wuhan, China) were added to each well for 24 hours. Empty lentivirus vectors were used as negative controls.

Flow Cytometry

The differentiated T cells were incubated for 4 hours at 37°C and 5% CO_2 with 0.1 mg/ml of monensin (Sigma, Saint Louis, USA), 1 μg/ml of ionomycin (Sigma, Saint Louis, USA), 500 ng/ml of sparfloxacin (Sigma, Saint Louis, USA).Then cells were stained with FITC-IL-17A monoclonal antibody (Multiscience, Hangzhou, China) and APC-CD4 monoclonal antibody (Multiscience, Hangzhou, China). The flow cytometry was performed in Cytek Athena system (Cytek, San Francisco, USA). Flow Jo software was used to analyze the results.

Immunofluorescence

The induced T cells were fixed in 4% paraformaldehyde (PFA) for 10 minutes, washed with PBS, permeated with PBS+0.2% Triton X-100 (PBTX) for 10 minutes, then blocked in 10% goat serum for 1 hour. The cells were incubated overnight at 4 °C with Rabbit anti-RORγt (GeneTex, Irvine, USA). The following day, the cells were washed with phosphate-buffered saline (PBS) containing 0.3% Triton X-100, blocked in 10% goat serum (GS) for 1 hour and then incubated at room temperature for 1 hour with the biotinylated universal secondary antibody and horseradish peroxidase-labelled streptomycin-avidin, The images were collected under fluorescence microscope after the slides were sealed with fluorescence quenching agent.

Statistical analysis

Statistical analysis was performed using SPSS 17.0. The data was expressed as a mean ± standard deviation. To compare the difference between two groups or multi-groups, t-test and one-way ANOVA were performed and LSD was used for post hoc test. $P < 0.05$ was considered significant.

Results

LncRNA-BG interacts with RORγt and inhibits the expression of IL-17

In our previous study, we obtained lncRNA-BG related to COPD by high-throughput screening. Through bioinformatics analysis, we found that the production of lncRNA-BG was from the gene STAT4. lncRNA-BG consisted of two parts: the first half came from the non-coding region of the STAT4 gene, and the second half came from the coding region of the STAT4 gene. After transcription, a mature lncRNA with a length of 486 nucleotides was formed (Fig. 1a). LncRNA-BG was predicted to interact with RORγt, and the interaction sites were the 50-100nt region of lncRNA-BG and the 350-450 amino acid region of RORγt protein. Meanwhile, LncRNA-BG was also predicted to interact with RORγt isoform, and the interaction sites were the 50-150 nt and 250-300 region of lncRNA-BG and the 400-500 amino acid region of RORγt protein (Fig. 1b). Using RNA pull down with biotin-labeled lncRNA-BG, we confirmed that lncRNA-BG interacted with RORγt in T cell extracts. This interaction still existed even in the presence of DNAase I or ethidium bromide (EB), suggesting that DNA was not involved the interaction between LncRNA-BG and RORγt protein (Fig. 1c). After the pGL3-IL-17, pcDNA3-RORγt and pcDNA3-BG plasmids were co-transfected into 293T cells in different combinations, we found that RORγt significantly promoted the activation of the IL-17 promoter, whereas lncRNA-BG inhibited the activity of the IL-17 promoter in a dose-dependent manner. It was proved that the lncRNA-BG gene played a direct role in inhibiting Th17 cell differentiation and Th17 related gene expression (Fig. 1d).

LncRNA-BG expression was down-regulated accompanied by up-regulation of IL-17 expression in COPD patients.

We isolated T cell from 16 clinical samples, including 6 normal controls and 10 COPD patients, and then measured the expression level of lncRNA-BG and IL-17A. Compared with the normal controls, the levels of IL-17 in cultured supernatant were significantly higher than those in the control (Fig. 2a). The mRNA levels of lncRNA-BG in COPD patients were significantly lower than those in the normal controls (Fig. 2b). Meanwhile, the RNA levels of IL-17A and RORγt in the COPD patients were significantly higher than those in the normal controls (Fig. 2c and fig. 2d).

LncRNA-BG expression was down-regulated accompanied by up-regulation of IL-17 expression in human $CD4^+$ T Cells

Human $CD4^+$ T lymphocyte cell line hut78 was cultured in vitro and induced into Th17 under 20 ng/mL of IL-6, 5 ng/mL of TGF-β, 25 ng/mL of IL-23, 5 ug/mL of anti-IFN-γ and 5 μg/mL of anti-IL-4 for three days

under the activation of 2 μg/mL of anti-CD3 and 5 μg/mL of anti-CD28. At the beginning of induction, the proportion of Th17 cells in hut78 cells was 0.091%. After two and four days of induction, the proportion of Th17 cells risen up to 5.71% and 10.2% respectively, indicating that the induction was effective (Fig. 3a-c). The levels of IL-17A in cell supernatant gradually increased with the increase of induction days (Fig. 3d). The results of Q-PCR showed that the mRNA levels of IL-17A and RORγt gradually increased, but lncRNA-BG gradually decreased and STAT4 also decreased (Fig.3 e-h). The results of Immunofluorescence showed that the level of RORγt protein gradually increased with the increase of induction days (Fig. 3i).

Down-regulation of lncRNA-BG promoted the differentiation of Th17 cells

Lentivirus carrying siRNA-BG could effectively infect human CD4+ T lymphocyte line Hut78, and the transfection efficiency reached 90%. Two days after lentivirus infection, hut 78 cells were induced into Th17 under the condition of Th17 differentiation in vitro. On the second day of induction, the RNA level of lncRNA-BG in knockdown group decreased significantly when compared with in the negative control group, but the mRNA level of IL-17A, RORγt, IL-17F and IL-21 increased significantly, and STAT4 showed no changes. On the fourth day of induction, all of the mRNAs mentioned above, including STAT4, were increased significantly in the knockdown group compared with the negative control group (Fig.4a-f). During the induction of differentiation into Th17 cells, the expression of RORγt in all three groups of cells increased gradually. The expression of RORγt protein in the knockdown group increased significantly when compared with the negative control group and blank control group, but there were no significant differences between blank control group and negative control group (Fig.4g). At the beginning of induction, the proportion of Th17 cells in T cells was 0.699%. The proportion of Th17 cells went up to 7.74% and 16.8% respectively after two and four days of induction (Fig. 4h).

Up-regulation of lncRNA-BG inhibited the differentiation of Th17 cells

On the other hand, we also up-regulated the expression of lncRNA-BG in human $CD4^+$ T lymphocyte line Hut78.Two days after lentivirus infection, hut 78 cells were induced into Th17 under the condition of Th17 differentiation in vitro. As shown in Fig.5A, lncRNA-BG increased significantly with the time of infection (Fig. 5a). LncRNA-BG overexpression decreased the level of IL-17A in hut78 supernatant (Fig. 5b). On the second day of induction, lncRNA-BG overexpression decreased the mRNA level of IL-17A, RORγt, IL-17F and STAT4 compare with negative control group, but IL-21 showed no change. There was no significant difference in the expression of these mRNAs between the negative control group and the blank control group. On the fourth and sixth day of induction, lncRNA-BG overexpression decreased all the level of mentioned mRNAs. Almost all mRNA levels decreased with increasing induction days (Fig. 5a-g). At the beginning of induction, the proportion of Th17 cells in T cells was 1.57%. After induction for two and four days, the proportion of Th17 cells reached 4.05% and 2.16%, respectively. It showed that the proportion of Th17 did not increase significantly after lncRNA-BG knockdown (Fig.5h).

Discussion

As an important subset of $CD4^+$ T lymphocytes, Th17 cells protected the mucosal surface mainly by secreting IL-17 and play a pivotal role in host defense against pathogens such as fungi and extracellular bacteria[23,24]. However, Th17 differentiation disorder produced excessive IL-17A could induce autoimmune tissue damages and inflammatory diseases[25,26].

IL-23 is a key cytokine for Th17 cell development and activation. Therefore, IL-23, IL-17A and their receptors are considered as drug targets for the treatment of Th17-related diseases[8]. In clinical trials, the antibodies of IL-23p19, IL-23p40, IL-17A, and IL-17RA have shown promising efficacy in the treatment of many autoimmune diseases including psoriasis, ankylosing spondylitis, and multiple sclerosis[27,28]. However, blocking IL-17A or IL-17RA is ineffective or even harmful in the treatment of Crohn's disease[29]. This may be due to insufficient blocking of a specific cytokine to inhibit Th17-mediated inflammation and several other cytokines produced by Th17 cells also, which play a key role in inflammation. However, the combined blocking of multiple cytokines will cause the dysfunction of other normal cells in the body. Recently, the antagonists of Th17 transcriptional regulator have been proposed as new potential therapies for Th17-mediated diseases. Th17 cell differentiation is regulated by an important set of transcription factors, including RORγt, STAT3, IRF4 and BATF[28]. Among these key transcription factors, RORγt is only highly expressed in Th17 cells[30], so RORγt may be an ideal therapeutic target. Several molecules targeting RORγt have been discovered, and they have shown efficacy in EAE, experimental colitis, experimental arthritis, and psoriasis-like skin inflammation models[31-33]. These studies suggest that RORdγt is clinically relevant as a therapeutic target for Th17-related diseases and is even a better therapeutic option than targeting cytokines or receptors.

LncRNAs are involved in cell development, differentiation, and growth by regulating all aspects of gene expression. For example, lncRNA XLOC _000261 negatively regulates the RORγt protein of Th17 cells in Crohn's disease[34]. LncRNA-MEG3 binds microRNA-17 as a ceRNA to regulate RORγt, ultimately affecting Treg/Th17 balance in asthma[35]. These studies suggest that lncRNAs can regulate the development and activation of TH17 cells to affect immune homeostasis by regulating the expression of RORγt protein.

In this study, we first demonstrated that lncRNA-BG interacted with RORγt and inhibited RORγt-induced differentiation of Th17 cells. In clinical sample and *in vitro* induction culture, we have demonstrated that down-regulation of lncRNA-BG expression is associated with up-regulation of RORγt and IL-17A. This result suggested that lncRNA-BG may negatively regulate Th17 differentiation. Then we artificially knocked down the expression of lncRNA-BG, and the results showed that the relative mRNA of Th17, including IL-17A, RORγt, IL-17F and IL-21 were all increased, and the proportion of Th17 cells was increased as well. Overexpression of lncRNA-BG resulted in the opposite effects. In addition, LncRNA-BG was derived from STAT4 gene. The RNA level of STAT4 was increased when lncRNA-BG was knocked down, and the RNA level of STAT4 was decreased when lncRNA-BG was overexpressed, indicating that lncRNA-BG may originate from STAT4, but give a feedback suppression to the expression of STAT4.

Conclusion

LncRNA-BG combined with RORγt protein could inhibit the activation of IL-17 promoter by RORγt protein, and ultimately inhibits Th17-related gene expression and Th17 cell differentiation. The expression mechanism of lncRNA-BG, its binding site to RORγt, its effect on the RNA level of STAT4 and whether the regulation of TH17 by lncRNA-BG is specific will be the focus of our next study. Consequently, this study demonstrates the potential of lncRNA-BG to be a target for clinical diagnosis, prognosis, phenotype and treatment of TH17-mediated diseases such as COPD.

Abbreviations

COPD: Chronic Obstructive Pulmonary Disease; AHR: airway hyperresponsiveness; MS:multiple sclerosis; RA: Rheumatoid arthritis; CD: Crohn's disease.

Declarations

Ethics approval and consent to participate

The study was approved by the Institutional Ethic Committee of Xiangya School of Medicine, Central South University.

Consent for publication

Not applicable.

Availability of data and materials

All datasets on which the conclusions of the manuscript rely were presented in the main paper.

Competing of Interests

The authors declare that they have no conflict of interest.

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Authors' Contribution

H H, XQ and MQ carried out the study. LQ performed the statistical analysis. OB and YT participated in the design of the study. All authors read and approved the submission.

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Not applicable.

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Figures

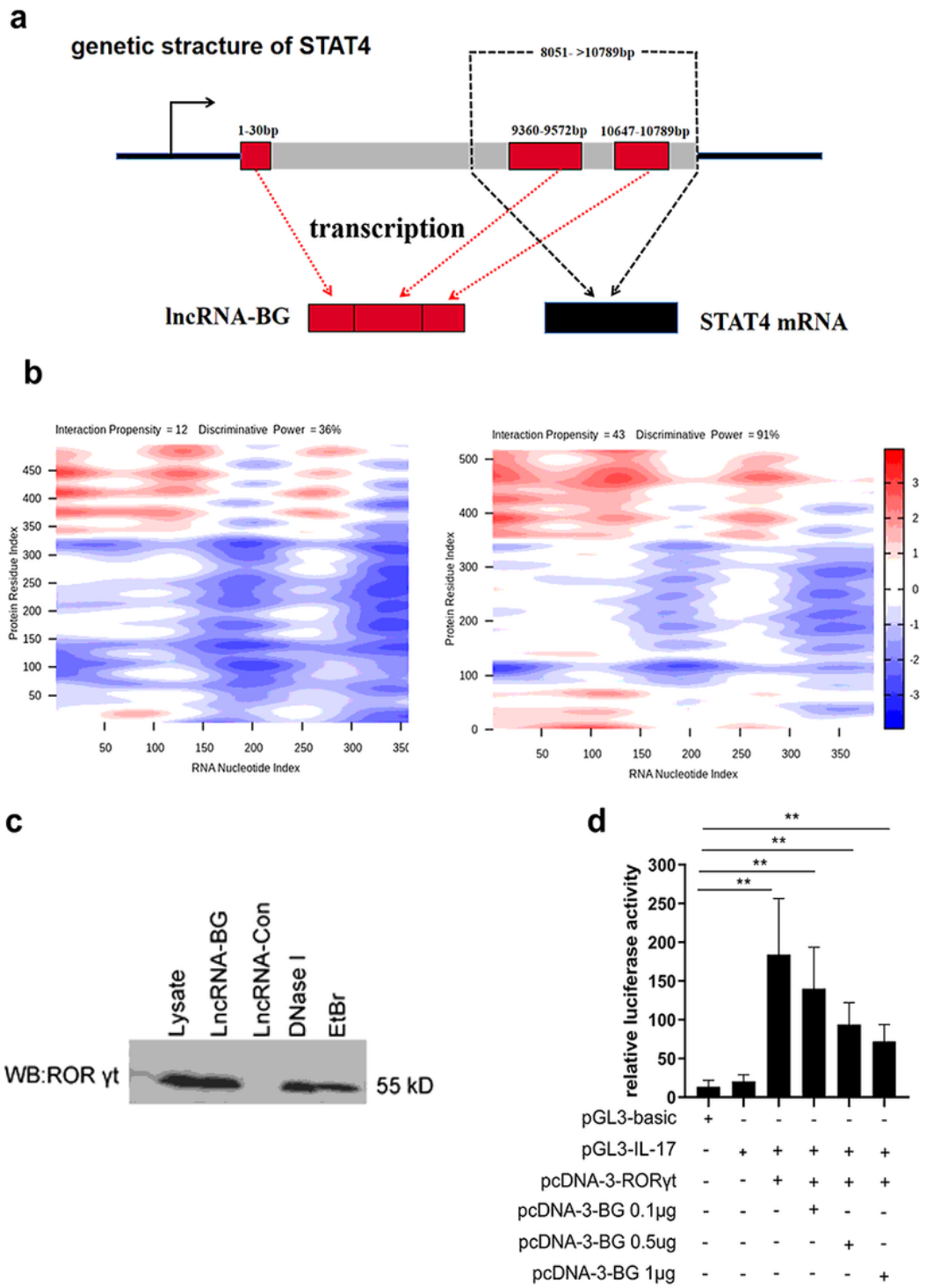

Figure 1

LncRNA-BG interacts with RORγt and inhibits IL-17 expression. a The schematic diagram of lncRNA-BG transcription. b Bioinformatics analysis predicted results of the interaction bewteen lncRNA-BG and RORγt protein. Left: the interaction bewteen lncRNA-BG and RORγt protein. Right:the interaction bewteen lncRNA-BG and RORγt protein isoform. c RORγt Protein was detected in extracts of the RNA pull-down by western blot. LncRNA-Con was used as negative control. d Dual luciferase reporter assay was used to

confirm the inhibition of lncRNA-BG during RORγt-induced activation of the IL-17 promoter. Three independent experiments were performed, and data are presented as mean ± SD.*P <0.05, **P <0.01, ***P <0.001.

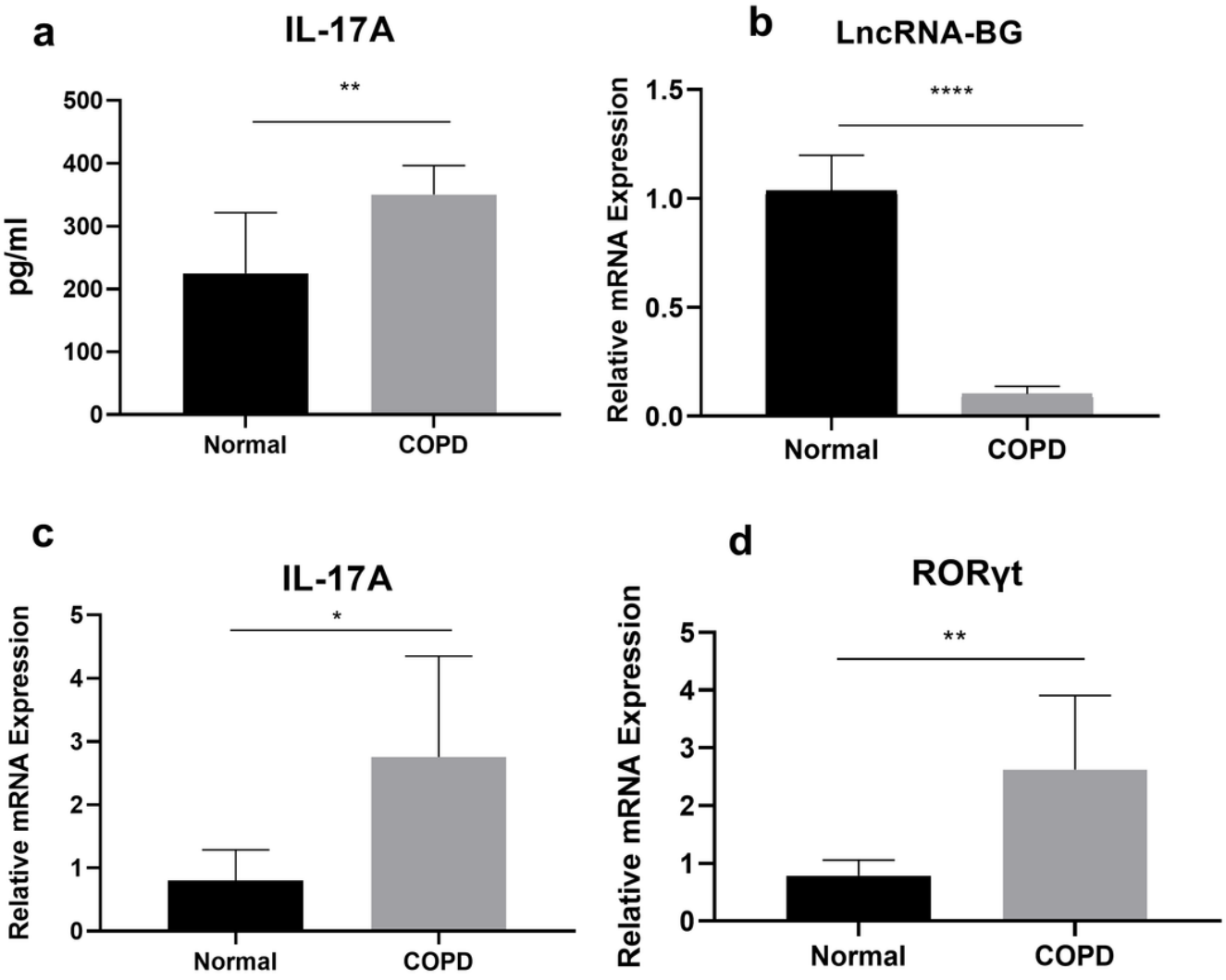

Figure 2

LncRNA-BG expression was down-regulated accompanied by up-regulation of IL-17 expression in COPD patients. a The release of IL-17A in peripheral T cells of COPD patients were detected using ELISA. b-d The mRNA expression of lncRNA-BG, IL-17A and RORγt in peripheral blood T cells of COPD were detected using Q-PCR. Three independent experiments were performed, and data are presented as mean ± SD. *P <0.05, **P <0.01, ***P <0.001.

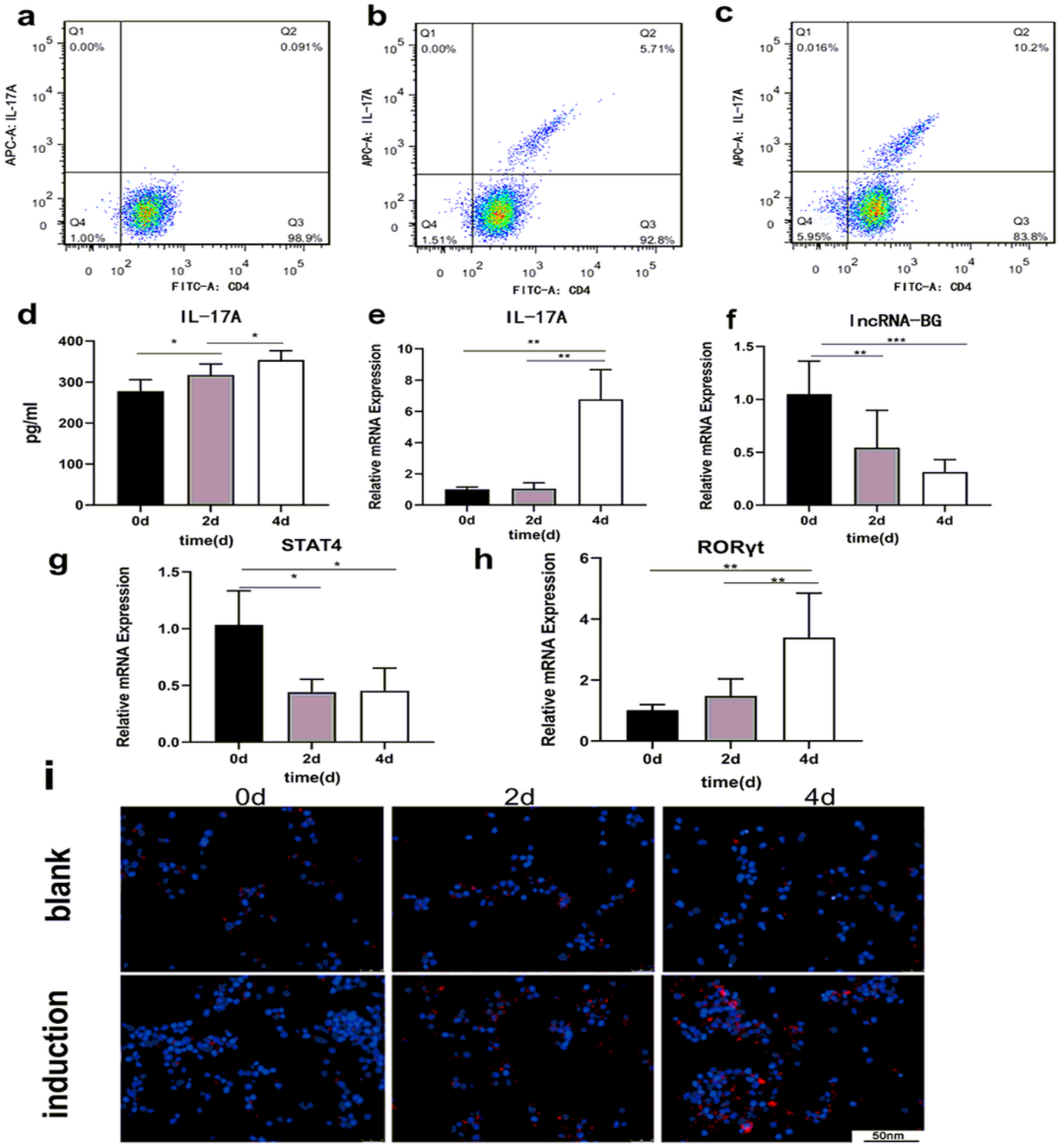

Figure 3

LncRNA-BG expression was down-regulated accompanied by up-regulation of IL-17 expression in Human Th17 Cells. a-c The proportion of Th17 cells was detected by flow cytometry on day 0, day 2, and day 4 of induced differentiation. d The levels of IL-17A in cell supernatant detected by ELISA. e-h The expression of IL-17A, lncRNA-BG, STAT4, and RORγt genes detected by q-PCR respectively. i The expression of

intracellular RORγt protein detected by immunofluorescence. Three independent experiments were performed, and data are presented as mean ± SD. *P <0.05, **P <0.01,***P <0.001.

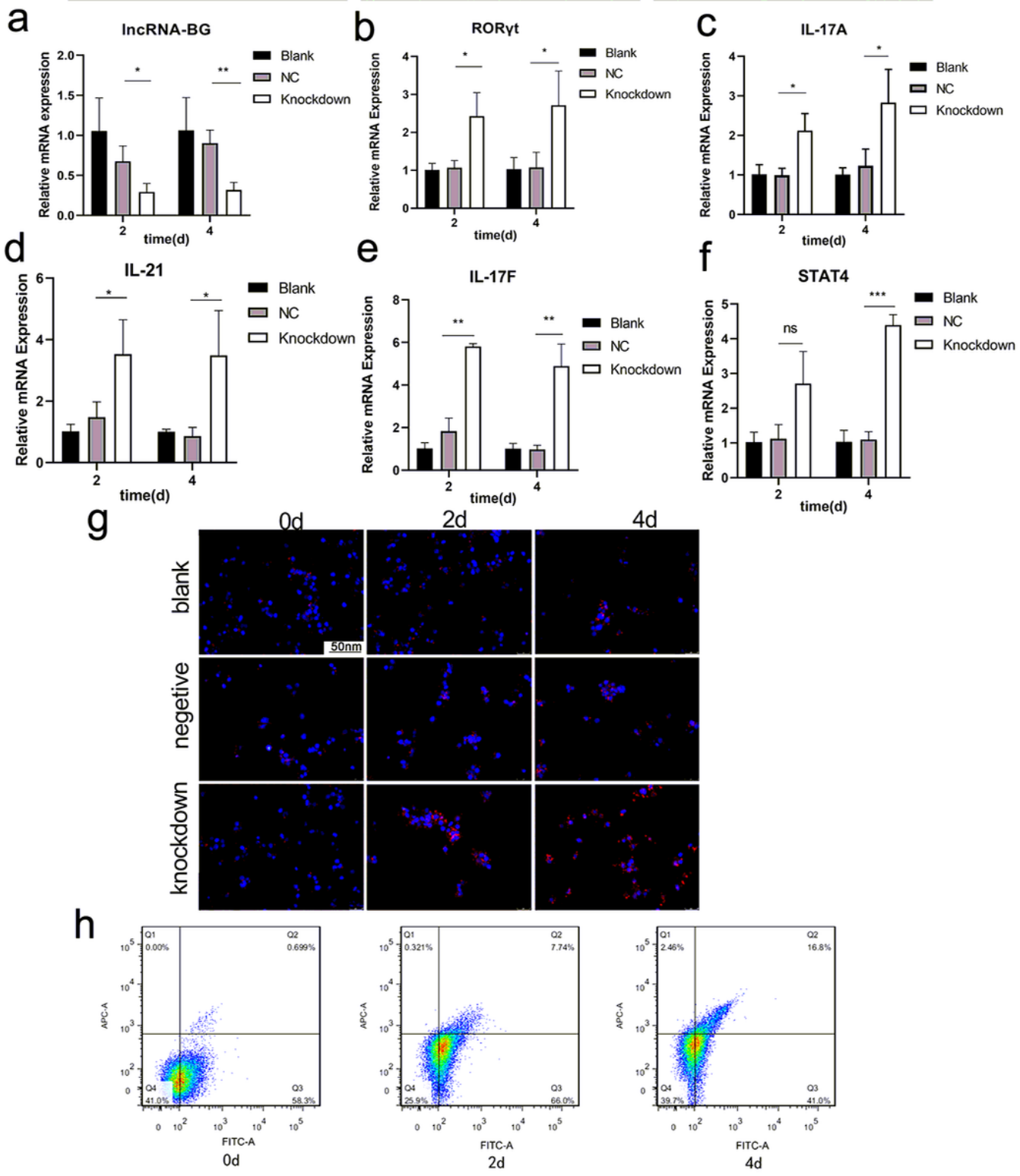

Figure 4

Down-regulation of lncRNA-BG promoted differentiation of Th17 cells. a-f The mRNA level of lncRNA-BG, STAT4, IL-17A, RORγt, IL-17F and IL-21 on day 2 and 4 of induction differentiation. g Immunofluorescence determined the protein expression of RORγt. h Flow cytometry determined the proportion of Th17 cells at

0, 2 and 4 days under the condition of TH17 differentiation. Three independent experiments were performed, and data are presented as mean ± SD. *P <0.05, **P <0.01,***P <0.001.

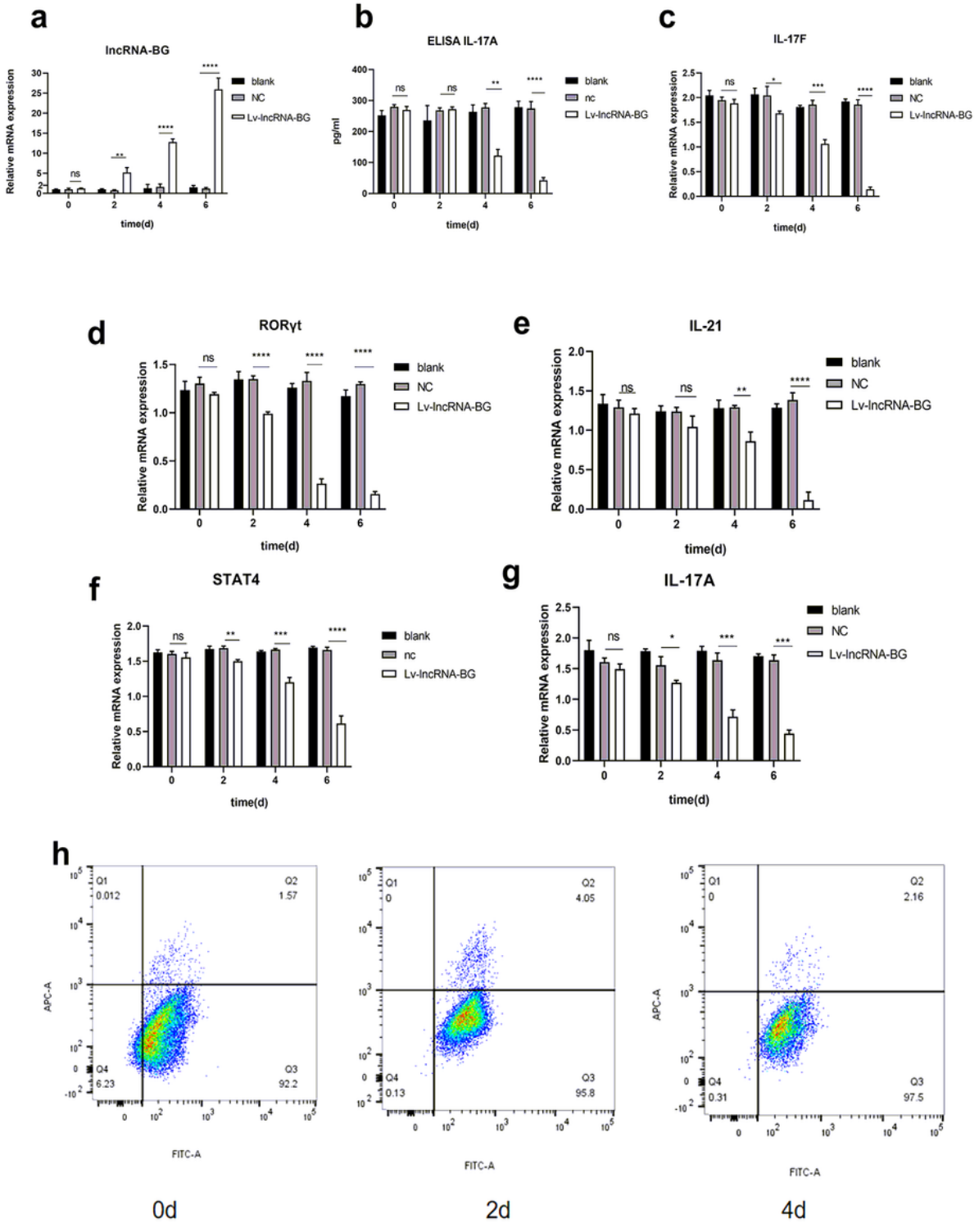

Figure 5

Up-regulation of lncRNA-BG inhibited the differentiation of Th17 cells. a The mRNA level of LncRNA infected with lentivirus. b The level of IL-17A in the supernatant after lentivirus infection. c-g The mRNA of lncRNA-BG, STAT4, IL-17A,RORγt, IL-17F and IL-21 at 2,4 and 6 days after induction. h The proportion of

Th17 cells induced by lncRNA-BG overexpression at day 0, day 2 and day 4.Three independent experiments were performed, and data are presented as mean ± SD. *P <0.05,**P <0.01,***P <0.001