

# B7-H2 Expression in Human Salivary Gland Epithelial Cells Affects Saliva Secretion in Primary Sjögren's Syndrome

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## Research Article

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# Abstract

Primary Sjögren's syndrome (pSS) is a chronic inflammatory autoimmune disease of exocrine gland. In pSS, permanent activation of the adaptive immune system is obvious. Lymphocyte co-stimulation plays an important part in inflammation and immunotherapy. Co-stimulator ligands (B7-H2) are significant costimulatory molecules. The interaction of B7-H2, with its sole receptor ICOS, promotes T cell differentiation, effector responses and activation. Our study found that B7-H2 is up-regulated in salivary gland, saliva and serum of pSS patients. B7-H2 expression in saliva have negatively correlated with saliva weight. Overexpression of B7-H2 into human salivary gland epithelial (HSGE) cells, increased the activity of p65 (phosphorylated at S536) and decreased the expression of AQP5. Furthermore, up-regulated B7-H2 induced apoptosis and inhibited proliferation in HSGE cell lines. These results suggest the expression of B7-H2 can decrease the secretion of saliva, increase the quantity of dental caries and reduce lifespan of patients of pSS.

## Introduction

pSS is a systemic autoimmune disease characterized by infiltration of periductal lymphocytes around the ducts in the lacrimal and saliva glands, with decreased secretory function (1). Compared with tears, saliva has advantages in terms of accessibility with no necessary of local stimulation. Saliva is a heterogeneous biofluid which is comprised of omics constituents' biomolecules. Saliva may be altered in response to various diseases, including pSS. Saliva has become a promising biological fluid for the detection of disease-specific biomarkers for pSS with potential role and clinical impact (2, 3). As we all known, the weight of saliva affects the number of caries. Some research found that the number of dental caries was related to the quality of life of patients (4). Therefore, the increase of saliva secretion of patients might reduce the caries problem and improve the quality of life of patients. However, few studies have focused on saliva secretion in the pSS.

T-cell differentiation and activation need two signals. T cell receptor (TCR) recognizes the antigen-MHC complex on antigen presenting cells (APC), which provides the first signal (5). The second signal necessary for T-cell activation and differentiation, is a costimulatory signal by the accessory molecules on APC (6, 7). B7-H2, one of the B7 family members, is expressed by macrophages (8, 9), activated B cells, epithelial cells and dendritic cells (10–12). B7-H2 with ICOS promotes T cell activation, differentiation and effector response, as well as B cell response (13). It has been shown that ICOS-mediated co-stimulation to T cells leads to secretion of interleukin (IL)-13, IL-2, IL-4 and suppression of  $\gamma$  interferon (IFN)- $\gamma$  and IL-10 (14). Apoptosis of SG epithelial cells is connected with misidentification of autologous antibodies to pSS SG epithelial cells and increasing the level of TNF- $\alpha$  (15). Some studies have shown that costimulatory molecules B7-H2 are involved in the pathogenesis of immune regulation abnormality in ITP (16). The function of B7-H2 has not been elucidated in pSS.

pSS is described as an autoimmune epithelial disease due to its relationship with various epithelial cells (17, 18). Hence, we chose HSGE cells for our research. Nuclear factor kappa B (NF- $\kappa$ B, p65) signaling is

related to the transcription of proinflammatory cytokines, such as TNF, monocyte chemoattractant protein 1 (MCP-1), IL-6 and IL-1 $\beta$  (19). However, the association of cytokines with pSS is not definite in terms of immunopathogenic and clinical aspects. It is not clear how HSGE cells are connected to the inflammatory micro-environment during the development of pSS. This study indicates that inhibiting the expression of B7-H2 may increase the saliva secretion and improve the quality of life in patients with pSS.

## **Materials And Methods**

### **Patients**

The study was conducted in the Affiliated Hospital of Nantong University and the experiment lasted for one year between September 2017 and September 2018. The patients' pSS diagnosis pact was in line with the 2016 American-European Consensus Group (AECG) SS classification criteria (20). We collected salivary gland biopsy specimens and assess pSS` pathological state of all participants. Donors who bears inflammatory disease, tumor, or infectious disease were removed from the sample groups. The experiment was approved by the medical ethics of the Affiliated Hospital of Nantong University (ethics approval number: 2017-K003). Informed consent was obtained by all participants. Table1 presented patient characteristics.

### **Saliva collection**

Unstimulated whole saliva (UWS): patients prohibited to stimulate the salivary flow. The subjects were asked for allowing the tongue to accumulate captain until enough saliva has pooled. Saliva was gathered for five minutes then it was dripped into a sample cup. From 9 to 12 a.m., saliva collections were performed in order to reduce the influence of circadian fluctuations. We centrifuged all the saliva samples at a speed of 4500g to remove debris and cells for 5 min at 4 °C (21). The samples were stored at -80 °C for further analysis.

### **ELISA**

A human ELISA kit (BSBIO, China) was used to quantify the levels of B7-H2 in saliva and peripheral blood. We incubated the serum and saliva samples on 96-well ELISA plates at a temperature of 37°C in an hour. Next, the result was observed by a microplate reader at a wave length of 450 nm. Each sample was repeated three times.

### **Immunofluorescence analysis**

We pre-soaked the salivary gland tissue in 4% paraformaldehyde and put them on ice for 3 hours. Then tissues were left at 4°C in 30% sucrose overnight. Those samples were put in OCT and cut by 5  $\mu$ m thickness. The salivary gland was blocked with 5% BSA at 37°C for 1 hour and then incubated with antibodies against B7-H2 (ab209262, Abcam, UK, 1:100) antibody at 4°C overnight, followed by staining with DAPI, goat anti-rabbit IgG (Vector Laboratories, USA). After that, secondary antibodies with red fluorescence dyes or green fluorescence dyes were stained (PE company, 1:3000).

## **Culture of HSGE cells**

HSGE cell line was purchased from SHANGHAI AOLU BIOLOGICAL TECHNOLOGY CO.LTD. Cells were cultured in DME/F12 with 1% streptomycin, penicillin and 10% fetal bovine serum. Cells were digested with 0.25% (w/v) trypsin/EDTA solution.

## **Flow cytometry**

Cell cycle analysis: we centrifuged and collected the HSGE cells in 95% ethanol at 4°C for a night. Next, cells were washed three times with PBS and were resuspended for 20 min with 100 µg/ml RNase (Sigma, USA). We stained cells with propidium iodide (PI) solution (Sigma, USA) for 30 minutes. Then cell were analyzed by the flow cytometry analyzer. We analyzed data with the ModFit LT system.

Cell apoptosis assays: the cells were detached, collected, washed with cold PBS. Then we resuspended the cells in 100µl Annexin V Binding Buffer with 5µl Annexin V- fluor647. In the dark, sequentially 5µl propidium iodide (PI) were added followed by incubation for 30 minutes at 37°C. The samples were measured by a BD FACSCalibur Cytometer.

## **Cell transfection**

HSGE cell ( $3 \times 10^5$ /well) were seeded in a six-well dish 12h before transfection. The cells were divided into three groups: the negative control group (NC: cells were transfected with 7.5µg lipo3000), up-regulated B7-H2 (7.5µg lipo3000+7.5µg B7-H2 PcDNA3.1(+) – Tat plasmid), down-regulated B7-H2 group (7.5µg lipo3000+7.5µg B7-H2-shRNA). B7-H2-shRNA and PcDNA3.1(+) – Tat plasmid were purchased from Shanghai GenePharma Co., Ltd. (China, Shanghai). When the cell confluence reached 40–60%, the HSGE cells were transfected. Lipo3000 was diluted with DME/F12 medium for transfection. Then shRNA and plasmids were added into the lipo3000 solution. The mixture stood still for 20 minutes before being dropped into each well. The cells were incubated with DME/F12 culture medium for 12 h. Then, the DME/F12 medium were replaced by complete culture medium with 36 hours of transfection. The cells were collected for upcoming experiments.

## **Western blot analysis**

The transfected cells were collected and lysed with RIPA buffer (Beyotime, USA) in addition with Phenylmethanesulfonyl fluoride (PMSF, Beyotime). Using bicinchoninic acid kits (BCA, Beyotime), protein concentrations were measured by spectrometer. 150µg protein samples each group were added into 10% SDS-PAGE wells for electrophoresis and then transferred into PVDF membranes. The PVDF membranes were soaked by BSA solutions to block the protein. Then the membranes were soaked with primary antibodies at 4°C overnight. The immunoblotting: anti-B7-H2 (Abcam), anti-AQP5 (Abcam), anti-p-p65 (Abcam), anti-p65 (Abcam). The standard band density (GAPDH) was used as internal reference. The experiment was repeated at least three times.

## **Statistical analysis**

SPSS 19.0 software and One-way ANOVA were performed to calculate *P* value of repeated experiments. All values represented as number (percentage), the mean  $\pm$  standard deviation (SD), median (IQR). \**P*>0.05 was considered to have no statistical significance. Every experiment replicates at least three times.

## Results

### Characteristics of controls and pSS patients (Table. 1)

The features of 95 pSS patients and 68 controls are summarized in Table. 1. Compared with controls, the expression of RF and anti-SS-A/RO antibodies were higher in pSS patients. Meanwhile, the ESSDAL, ESSPRI, the focus score of salivary gland biopsy and the number of dental caries in pSS patients tended to be excessive.

### The difference of saliva weight, ESPRI, number of caries between pSS patients and healthy controls (Table. 2)

Compared with controls in Table2, saliva weight was significantly lower in pSS patients ( $0.77\pm 0.05$  vs  $1.40\pm 0.07$ ; *P*<0.001). Compared with controls, the number of caries was significantly lower in pSS patients ( $3.75\pm 0.64$  vs  $0.10\pm 0.06$ ; *P*<0.001).

### B7-H2 expression in serum, saliva and salivary gland of controls and pSS patients (Fig. 1)

Compared with controls, the levels of B7-H2 in peripheral blood (*P*<0.05) and saliva (*P*<0.01) elevated in pSS patients (Fig.1 a, b). Immunofluorescence analysis of pSS patients' salivary gland tissues showed higher expression of B7-H2 (*P*<0.001) than healthy groups (Fig.1c). The positive ratio of B7-H2 in pSS patients' and controls' salivary gland was nearly 57.23% vs 30.36% (Fig. 1d). In summary, the expression of B7-H2 in pSS patients was markedly higher in controls (*P*<0.05).

### The relationship between saliva weight and the number of caries, ESSPRI, the expression of B7-H2 in saliva (Table. 3)

In pSS patients, saliva weight was significantly negative associated with the number of caries ( $r=-0.357$ , *P*=0.008) and ESSPRI ( $r=-0.24$ , *P*=0.035). It also unexpectedly showed that saliva weight was negatively associated with the expression of B7-H2 ( $r=-0.380$ , *P*=0.001) in pSS patients.

### The impact of B7-H2 expression on HSGE cells apoptosis (Fig. 2)

We further detected cell apoptosis in the up-regulated B7-H2, down-regulated B7-H2, NC cells by Annexin V/PI double staining (Fig. 2a). The results showed that the total apoptosis rate of HSGE cells increased in the up-regulated B7-H2 group (*P*=0.017) and decreased in the down-regulated B7-H2 group (*P*=0.0086) compared with NC group (Fig. 2b).

### The impact of B7-H2 expression on HSGE cells proliferation (Fig. 3)

Cell proliferation was determined by flow cytometry (Fig. 3). Our data showed that all three groups experienced slowing growth period, logarithmic multiplication period and growth stationary period. The results of cell cycle distribution showed that the percentages of HSGE cell in G0/G1 phase of NC, down-regulated B7-H2 group and up-regulated B7-H2 group were  $57.14 \pm 2.752\%$ ,  $60.76 \pm 2.78\%$  and  $62.8 \pm 2.262\%$ , respectively. The S phase fraction in each group was  $23.59 \pm 1.332\%$ ,  $20.35 \pm 3.151\%$  and  $24.01 \pm 3.233\%$ , respectively. The G2/M phase fraction in each group was  $16.66 \pm 2.084\%$ ,  $17.34 \pm 1.302\%$  and  $9.85 \pm 0.585\%$ , respectively (Fig. 3a). Through statistical analysis (Fig. 3b), there was significant difference between NC and up-regulated B7-H2 in G2/M phase. However, no significant difference was found between NC and down-regulated B7-H2 in G0/G1, S and G2/M phase, respectively. Thus, we could conclude that up-regulated B7-H2 has apparent effect on HSGE cell proliferation.

#### **The impact of B7-H2 on relative protein expression (Fig. 4)**

To further determine the relationship between B7-H2 expression and saliva, we detected the expression levels of AQP5 and B7-H2 (Fig. 4a). Up-regulated B7-H2 and down-regulated B7-H2 were established by transfecting plasmids into HSGE cell lines. Western blot assay showed that the expression of AQP5 was markedly decreased with up-regulated B7-H2 ( $P < 0.001$ ) and elevated with down-regulated B7-H2 ( $P < 0.001$ ). Recent studies have shown that B7-H3 accelerates metastasis and invasion of pancreatic cancer cells via the TLR4<sup>-</sup>/NF- $\kappa$ B pathway (22). In adaptive and innate immunity, p65 plays an important role as a transcription factor. However, the mechanism regarding B7-H2 and p65 has not been clearly understood in pSS. We tested the expression of p65 and p-p65. We unexpectedly found that up-regulated B7-H2 increased the expression of p65 and p-p65 and down-regulated B7-H2 decreased the expression of p65 and p-p65 (all  $P < 0.05$ ).

## **Discussion**

pSS is a systemic autoimmune disorder with periductal lymphocytes infiltration in lacrimal and saliva glands, which leads to low secretory function, dry mouth and eyes. pSS also cause other symptoms such as early tooth loss (23, 24), atypical and/or severe dental caries (25–29), occurrence of oral candidiasis and atrophic oral mucosal lesions or lobulation of the tongue (30). In addition, the sequelae, such as changes in sense of taste, dysphagia (31, 32) and difficulty in eating and speaking, burning sensation, combined with chronic fatigue (33), may reduce patient's quality of life. However, cellular and humoral mechanisms underlying pathogenesis of Sjögren's syndrome are still not fully elucidated.

Saliva is indispensable in maintaining the oral health and homeostasis, by lubricating and moistening the oral tissues to aid in swallowing, chewing, speech, and tasting (34). Saliva also helps initiate digestion. In addition, maintenance of oral health largely depends on saliva's cleansing actions and intrinsic antipathogenic characteristics (35). The salivary dysfunction in pSS affects the patients' life quality and oral health (36, 37). Two of five pSS patients may have poor teeth, presumably because of the various tooth extractions, and they also experienced a higher risk of dental caries than people in general. In our study, we found that there is a strong difference in the weight of saliva, the number of caries and ESSPRI

between controls and pSS patients. There are reports showing that dental caries affects the quality of life of patients (4, 38).

Accumulated evidence suggests that ICOS is instrumental in T cell-driven multi-organ inflammation in autoimmune diseases (39). B7-H2 was found to be expressed not only in APCs, but also in SGECs in pSS. These SGECs play an important role in the differentiation of naive CD4 + T cells into follicular helper T cells by secreting IL-6. Interactions between B7-H2 on SGECs and ICOS on follicular helper T cells results in enhanced IL-21 secretion by follicular helper T lymphocytes (40). Our study also proved that B7-H2 is expressed in HSGE cell. The expression of B7-H2 was distinct between pSS patients and controls. In pSS patients' saliva, there is a negative correlation between the expression of B7-H2 and the weight of saliva. It is proposed that up-regulated B7-H2 affects saliva secretion in pSS. Recently, it has been reported that the ICOS-B7-H2 interaction helps immune regulation and promotes the activation and expansion of regulatory T (Treg) cells (41–43). The expression of B7-H2 in pSS patients' salivary gland is higher than those in controls' salivary gland.

Whether the ICOS-B7-H2 signaling pathway affects the secretion of saliva is still remained for us to answer. Recent study indicates that poor saliva secretion is partly due to abnormal distribution of AQP5 in HSGE cells (44). Moreover, the expression of AQP5 is influenced by apoptosis-related molecules and inflammatory cytokines. Through in *vitro* experiments, we found that B7-H2 expression accelerated apoptosis of HSGE cells and reduced cell proliferation ratio with decreased AQP5 expression. We hypothesize that overexpression of B7-H2 leads to cellular inflammatory responses and then affects saliva secretion.

P65, a member of nuclear transcription factor, participates substantially in regulating multiple important pathologic processes, such as apoptosis, inflammation and various autoimmune diseases (45, 46). P65 activity is involved in immune regulation and abnormal p65 activation could result in tumorigenesis and inflammatory responses. Some other evidence also proved that increment of p65 in glandular epithelial cells could induce Sjögren's-like features (47). However, B7-H2 has not been studied in pSS. Through western blot assays, we unexpectedly found that up-regulated B7-H2 promoted the expression and activation of p65. The specific mechanism of action will be the focus of our following research.

## Conclusions

In summary, B7-H2 was highly expressed in pSS patients, which was negatively correlated with saliva weight in pSS patients. Moreover, up-regulation of B7-H2 could induce apoptosis and inflammation, and decrease the expression of AQP5 in HSGE cells. Inhibiting B7-H2 might promote saliva secretion and improve quality of life in pSS patients.

## Declarations

### Ethics approval and consent to participate

Written informed consent for the use of saliva, serum samples and salivary gland was obtained from all pSS patients. The study was approved by the ethical committees of the Affiliated Hospital of Nantong University, Nantong, China (approval number 2017-K003).

### **Consent for publication**

Not applicable.

### **Availability of data and materials**

All data generated or analyzed during this study are included in this published article if additional information. If any additional information is required it may be obtained by request with the corresponding author.

### **Competing interests**

The authors do not have conflict of interests to be declared.

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### **Authors' contributions**

JJ and ZX conceived and designed the experiments; PL, YJ, RZ performed the experiments; PL analyzed the data and contributed reagents/materials/analysis tools, wrote the paper. All authors read and approved the final manuscript.

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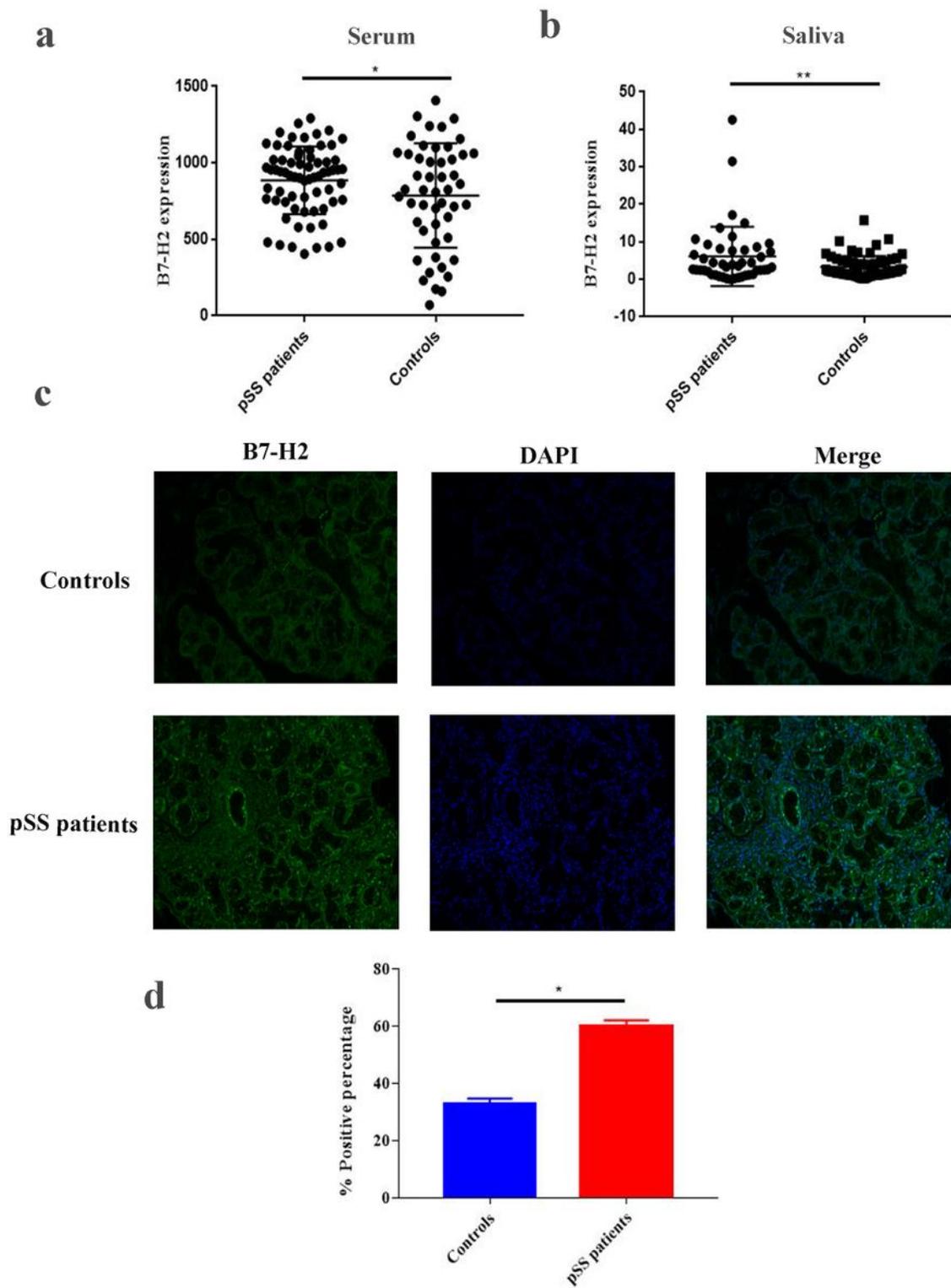
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## Tables

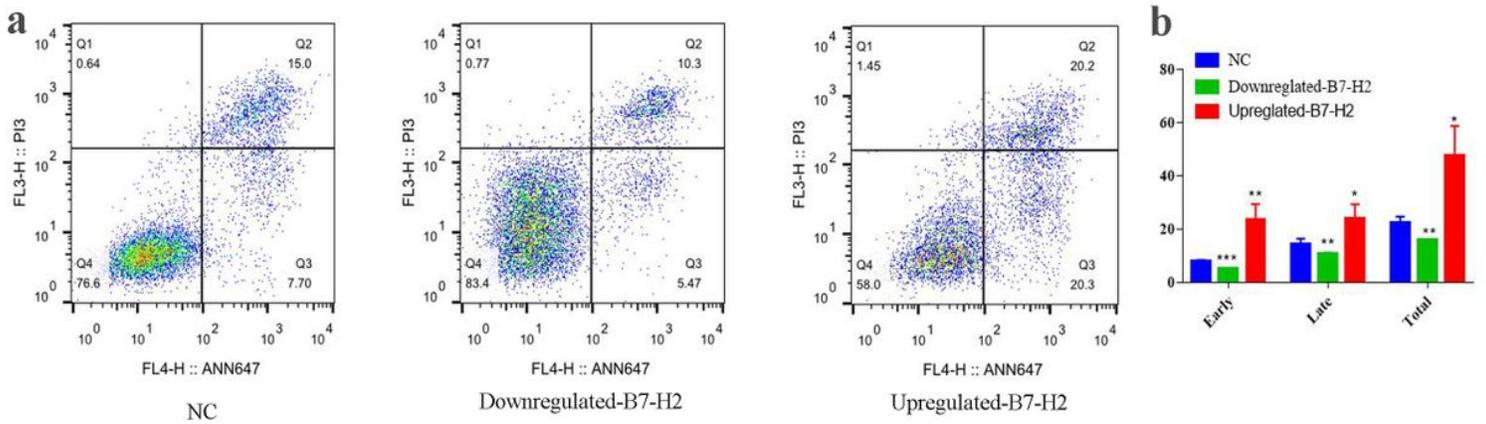
Due to technical limitations, tables 1,2 & 3 are only available as a download in the Supplemental Files section.

## Figures



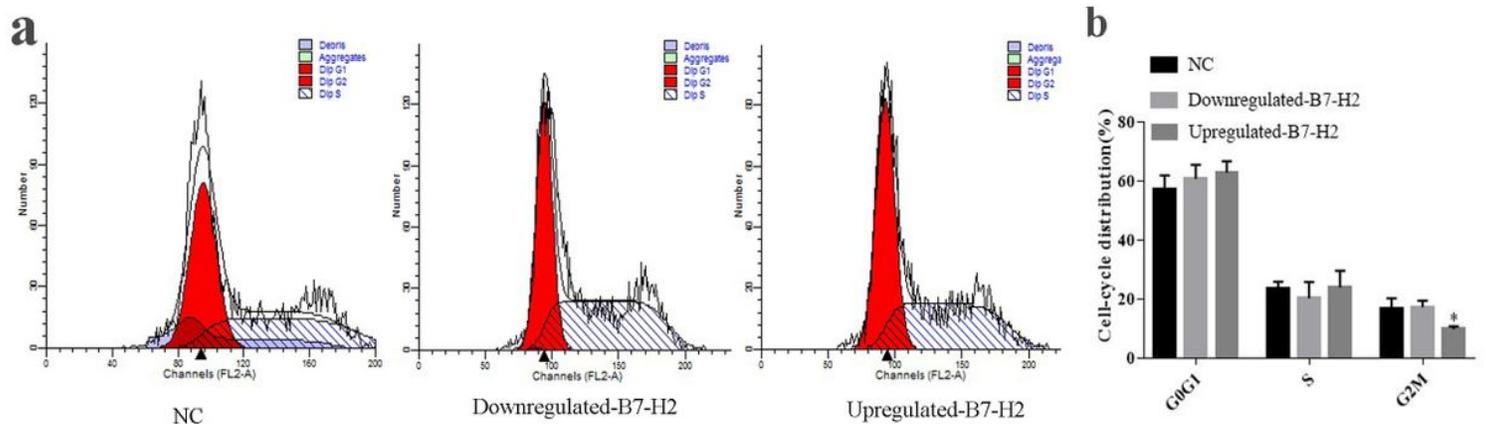
**Figure 1**

B7-H2 levels in serum, saliva and salivary gland in controls and pSS patients. (a) The pSS patients (n=69) had higher levels of B7-H2 in serum compared to controls (n=50). (b) The pSS patients (n=99) had higher levels of B7-H2 in saliva compared to controls (n=68). (c) The expression of B7-H2 in pSS patients was significantly higher than that in controls by IF in salivary glands tissues. (d) Quantitative analysis of the results of IF. \*\*\*P<0.001, \*\*P<0.01, \*P<0.05 vs. the controls.



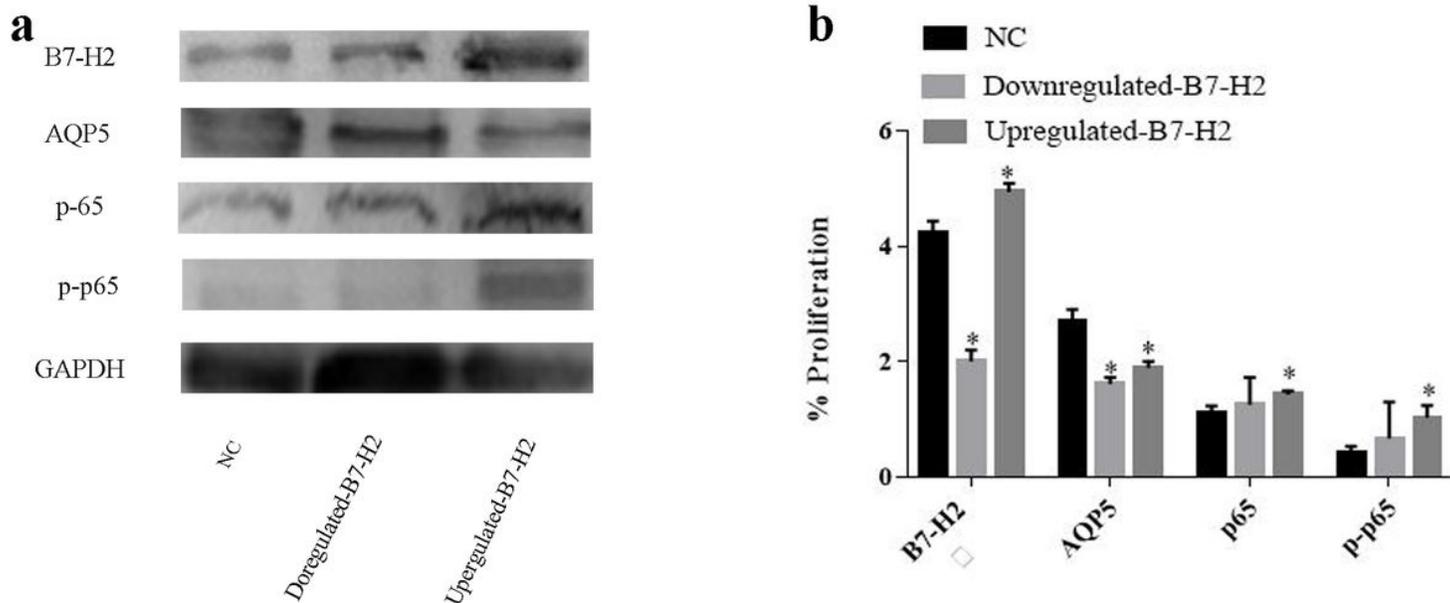
**Figure 2**

The impact of B7-H2 on HSGE cell apoptosis The cells were transfected for 48h before analysis. (a) The apoptosis rates of NC, down-regulated B7-H2, up-regulated B7-H2 groups of HSGE cells were analyzed. (b) Quantitative results of the total percentages of apoptotic cells in the NC, downregulated B7-H2, up-regulated B7-H2 groups. \* $P < 0.05$  vs. the NC group.



**Figure 3**

The impact of B7-H2 on HSGE cell proliferation (a) Cell proliferation of the NC, down-regulated B7-H2, up-regulated B7-H2 group were analyzed. (b) Quantitative analysis of the transfected cell proliferation. \* $P < 0.05$  vs. the NC group.



**Figure 4**

The impact of B7-H2 on relative protein expression of inflammatory factors We transfected the cells for 48h before analysis. (a,b) The levels of B7-H2 and other proteins of different B7-H2 influenced groups were quantified and assessed by Western blot analysis. GAPDH was probed as a positive control. \*P<0.05 vs. the NC group.

## Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

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