Hydrogen Sulfide Inhibits Abnormal Proliferation of Lymphocytes via AKT/GSK3β Signal Pathway in Systemic Lupus Erythematosus Patients

Yanfang Han a, Fanqin Zeng a, Guozhen Tan a, Chuntao Yang b, Hongfeng Tang c, Yi Jin Luo a, Jianqiang Feng d, Hui Xiong a, Qing Guo a

a Department of Dermatology, Sun Yat-sen Memorial Hospital, Sun Yat-sen University; Guangzhou;
b Department of Physiology, Guangzhou medical University, Guangzhou; c Department of Dermatology, The first people’s hospital of Shunde, Foshan; d Department of Physiology, Zhongshan School of Medicine, Sun Yat-sen University, Guangzhou

Key Words
AKT • GSK3β • Cell cycle • Hydrogen sulfide • Lymphocytes • Systemic lupus erythematosus

Abstract
Background/Aim: The abnormal activation of the AKT/GSK3β signal pathway in lymphocytes from systemic lupus erythematosus (SLE) patients plays an important role in the pathogenesis of the disease. Recently, hydrogen sulfide (H₂S) has been recognized as a crucial gaseous signaling molecule, involved in regulation of cell proliferation. However, the role of H₂S in regulating the abnormal activation of lymphocytes from SLE patients has not been established. This study was conducted to investigate the effect of H₂S on lymphocytes and to explore the mechanisms involved. Methods: The lymphocytes were isolated from SLE patients with or without renal disease and healthy controls. The cells were treated as indicated in each experiment. Cell viability was analyzed by CCK-8. Cell cycle distribution was determined by flow cytometry. Western blot was used to detect the expression of phosphorylated AKT (ser473), GSK3β (ser9) and CDK2, p27Kip1 and p21WAF1/CIP1. Results: Our findings showed that proliferation of lymphocytes was stimulated following treatment with NaHS (a H₂S donor) at low NaHS concentrations (<1mM) but inhibited at high NaHS concentrations (>2mM). Similar results were observed using GYY4137, which is a slow-releasing H₂S donor. Pretreatment of lymphocytes from SLE patients with NaHS at high concentrations prior to exposure to phytohemagglutinin (PHA) significantly attenuated proliferation, evidenced by decrease in cell viability and S phase distribution of cell cycle. Pretreatment with NaHS decreased PHA-induced expression of CDK2, phosphorylation levels of AKT (ser473) and GSK3β (ser9) and increased the expression of p27Kip1 and p21WAF1/CIP1. Moreover, pretreatment with NaHS blunted the stimulation of SLE lymphocyte proliferation by GSK3β inhibitor lithium chloride. Conclusion: These results demonstrate that H₂S inhibits the abnormal activation of lymphocytes from SLE patients through the AKT/GSK3β signal pathway.

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Introduction

Systemic lupus erythematosus (SLE) is an autoimmune disease characterized by various immunological abnormalities. Although the cause of SLE remains incompletely understood, the abnormal activation and proliferation of lymphocytes have been proven important in the pathogenesis of SLE [1].

It has been established that cell proliferation depends on the interactions between cell cycle regulatory proteins, such as cyclin-dependent kinases (CDKs), p27Kip1 and p21WAF1/CIP1. Deficient expression of these proteins in T-cells can promote the development of lupus in mice [2]. The PI3K/AKT/GSK3β signal pathway is crucial in regulating autoimmune system [3]. The increased AKT activation plays an important role in lymphoproliferation and results in lymphadenopathy and splenomegaly with accumulation of CD4+ and CD8+ T-cells, as well as B cells in such transgenic mice [4, 5]. We previously found that the proportions of lymphocytes in patients of SLE or RSLE with renal disease (RSLE) were reduced in the G1/G0 phase and elevated in the S phase after phytohemagglutinin (PHA) treatment. The phosphorylation levels of AKT (ser473) and GSK3β (ser9) were increased while the expressions of p27Kip1 and p21WAF1/CIP1 were decreased in lymphocytes from the patients [6]. It indicates that the AKT/GSK3β signal pathway closely relates to the abnormal activation and proliferation of lymphocytes in SLE patients.

In recent years, H2S has been considered as a biologically relevant signaling molecule with potential roles in several physiological processes, such as, anti-inflammation, promoting cellular signals, protecting vascular endothelium and so on [7-10]. Previous evidence indicates that H2S has biphasic effects on T cell proliferation [11, 12], stimulating T cell proliferation at low concentrations while inhibiting at high concentrations. Little work has been focused on the impact of H2S to the proliferation of lymphocytes from SLE patients, and whether the AKT/GSK3β signal pathway was involved in such process. This study aims to clarify the effect of exogenous H2S on the abnormal activation and proliferation of lymphocytes from SLE patients, and explored its potential relation with AKT/GSK3β signal pathway.

Materials and Methods

Reagents

Ficoll-Paque density gradient centrifugation medium was bought from Amersham Pharmacia Biotech (Uppsala, Sweden). RPMI 1640 medium and fetal bovine serum were purchased from Gibco (Grand Island, NY, USA). The antibody against cyclin-dependent kinase-2 (CDK2) was from BD Transduction Laboratories (San Diego, CA, USA). Antibodies against p27Kip1, p21WAF1/CIP1, total AKT, Ser473-phosphorylated AKT, total GSK3 or Ser9-phosphorylated GSK3β, and horseradish peroxidase (HRP)-conjugated secondary antibodies were purchased from Cell Signaling Technology (Beverly, MA, USA). RNase A, propidium iodide (PI), monoclonal rabbit anti-GAPDH antibody, phytohemagglutinin (PHA) and GY4137 were purchased from Sigma-Aldrich Co., (St. Louis, MO). Cell Counter Kit-8 (CCK-8) was purchased from Dojindo Laboratory (Kyushu, Japan). Lithium Chloride (LiCl) was purchased from MP Biomedicals (California, USA). Enhanced chemiluminescence (ECL) detection system was purchased from Applygen Technologies (Beijing, People’s Republic of China).

Patients, controls and blood samples

SLE patients were recruited from the outpatient clinics and inpatient services at the Sun Yat-sen Memorial Hospital, Sun Yat-sen University (Guangzhou, China). The diagnosis of SLE was established based on the 1997 revised American Rheumatism Association criteria. Renal disease was defined as proteinuria of ≥ 0.5 g/day. The patients were divided in two groups: 10 SLE patients with renal disease (RSLE group) and 9 SLE patients without renal disease (SLE group). All patients were diagnosed with SLE for the first time or without treatment with glucocorticoid or immunosuppressive agent over one month. Ten sex- and age-matched, healthy Chinese volunteers were recruited as controls. Twenty-five milliliters of heparinized venous peripheral blood was collected from each patient and control. The protocol of this study was
approved by the Clinical Research Ethics Committee of Sun Yat-sen University and signed informed consent was obtained from the healthy volunteers and the patients.

**Cell culture**

Peripheral blood lymphocytes (PBL) were isolated from heparinized venous blood by Fiocell-Paque density gradient centrifugation. The cells were washed twice with PBS and cultured in RPMI 1640 supplemented with 10% FBS, 20 mM HEPES (Gibco) and a combination of 100 U/ml penicillin and 100 μg/ml streptomycin at 37°C in a humidified incubator under an atmosphere of 5% CO₂. After cultured for 4 hours, the suspension cells were collected and used in the following experiments, which were PBL including T cells and B cells.

**Measurement of H₂S levels.**

H₂S levels were measured as previously described [13]. Amperometric H₂S sensors (ISO-H₂S-100, World Precision Instruments, Sarasota, FL, USA) were used for the real-time measurement of dissolved H₂S concentration in the medium.

**Flow cytometry**

PBL from patients and controls were centrifuged at 1,500 rpm for 5 min. The resulting pellets were washed with 1 ml of ice-cold PBS and fixed with 70% ethanol at 4°C for 24 h. Prior to staining, the cells were washed twice with PBS. The cell pellets were then stained with staining buffer (PBS containing 50 μg/ml propidium iodide, 10 μg/ml RNase A, 0.1% sodium citrate and 0.1 Triton X-100) for 30 min at room temperature in the dark. DNA content was analyzed by flow cytometry (Becton Deckinson, USA).

**Proliferation assays**

The proliferation of PBL obtained from the patients and controls was assessed using the CCK-8 assay after cells were treated with PHA, NaHS or a GSK-3β inhibitor (LiCl) as indicated in each study. Briefly, cells were seeded at 100,000 cells per well in 96-well plates and treated with indicated conditions for 48 h, and 10 μl CCK-8 solution was added to each well, and the cells were incubated for a further 4 h at 37°C. Absorbance was assessed at 450 nm with a microplate reader (Bio-Tek, USA). Vehicle controls for the respective inhibitors were tested simultaneously. The mean optical density (OD) of three wells in each group was used to calculate percentage of cell proliferation as follows:

\[
\text{Cell proliferation (\%)} = \left( \frac{\text{OD treatment group} - \text{OD blank}}{\text{OD control group} - \text{OD blank}} \right) \times 100
\]

**Western blot analysis**

At the end of treatments, PBL were washed once with PBS and lysed with solubilization buffer (50 mM Tris-Cl, 150 mM NaCl, 0.02% NaN₃, 1% Triton, X-100, 5 mM EDTA, 0.1 mM PMSF, 1 mM NaVO₃, 20 μg/ml aprotinin and 20 μg/ml leupeptin). After centrifugation, the supernatants were collected, and the protein concentration was determined by a BCA protein assay kit (Bio-Rad, Hercules, CA). Equal amount of proteins were loaded and separated by 10% SDS-PAGE. The separated proteins were transferred onto PVDF membranes. After blocking with TBST containing 5% nonfat milk for 1 h, each membrane was incubated with different primary antibodies, HRP-conjugated secondary antibodies, and visualized by enhanced chemiluminescence. The results were qualified by densitometry.

**Statistical analysis**

Statistical comparisons were performed using independent-samples t tests or one-way analysis of variance (ANOVA) by SPSS 13.0 (SPSS, USA). For all experiments, data are presented as means ± SD. P-values less than 0.05 were considered significant.

**Results**

\( \text{H}_2\text{S had biphasic effects on the proliferation of lymphocytes} \)

It is reported that H₂S regulates the proliferation and apoptosis of many kinds of cells [7]. To assess the effect of exogenous H₂S on lymphocytes from SLE patients, lymphocytes
were isolated from healthy controls, SLE and RSLE patients respectively, then treated with increasing concentration of NaHS (a H₂S donor, including concentrations of 0.25, 0.5, 1, 2, 4 and 8.0 mM). The concentrations of H₂S released by 0.25-8mM NaHS at different time points were measured and shown as in Fig. 1. We observed that when treated by NaHS in low concentrations (0.25-1mM), the cell viability of lymphocytes from SLE and RSLE patients were increased, but decreased in higher concentrations (≥2mM), obviously at the concentration of 4 mM (Fig. 2B, C). Cell viability of lymphocytes from healthy controls slightly increased with the treatment of NaHS in low concentration, but decreased significantly at the concentration of 2 mM (Fig. 2A). Lymphocytes from SLE and RSLE patients proliferated obviously after treated with PHA. Pretreatment with NaHS at 4 mM inhibited the cell viability of PHA-induced lymphocytes from SLE and RSLE patients. However, the cell viability was harmed with pretreatment of NaHS at 8 mM (Fig. 2). Interestingly, we observed similar results in the experiment using GYY4137, which is a slow-releasing H₂S donor (Fig. 3). The results indicated that H₂S in different concentration showed different effects on proliferation of lymphocytes from both healthy controls and lupus patients. The concentration which was needed to inhibit the abnormal proliferation of lymphocytes from lupus patients was higher than that needed for the lymphocytes from healthy controls.

**H₂S pretreatment changed cell cycle distribution of PHA-induced lymphocytes from SLE patients**

Lymphocytes from patients and healthy controls were distributed into three cell cycle phases (G₁/G₀, S and G₂/M) using flow cytometry. As showed in Table 1 and Fig. 4, after treatment with PHA for 48 hours, the percentage of lymphocytes from SLE and RSLE increased significantly in S phase while decreased in G₂/M phase compared with those from healthy controls. In addition, lymphocytes from RSLE in S phase increased more significantly than those from SLE after being stimulated with PHA. But pretreatment with NaHS (at 4 mM) induced lymphocytes accumulation at the G₂/M cell cycle checkpoint. The populations in G₂/M, and S phase of lymphocytes in the pretreatment with NaHS group were similar to the untreated group respectively. Additionally, we found that NaHS at 4 mM didn’t induce apoptosis of lymphocytes (Fig. 5). These observations indicated that H₂S altered the abnormal cell cycle distribution of PHA-induced lymphocytes from SLE and RSLE patients.

**H₂S altered expressions of CDK2, p27Kip1 and p21WAF1/CIP1 in stimulated lupus lymphocytes**

The interaction between CDKs and CDK inhibitors (CDKI) governs the G₁/S transition. It has been proven that the upregulation of CDK2 and downregulation of p27Kip1 and p21WAF1/CIP1 correlated with the abnormal proliferation of lymphocytes from lupus patients in our previously study[6]. Therefore we next assessed whether H₂S pretreatment would affect the expression of these proteins in lymphocytes while changing the distribution of cell cycle. As shown in Fig. 6, after treatment of lymphocytes from RSLE patients with PHA for 48 hours, the expression of CDK2 increased while the expressions of p27Kip1 and p21WAF1/CIP1 decreased. However, the expressions of these three proteins in NaHS pretreatment groups (both with and without PHA treatment) showed no obvious differences to those in the untreated group.
lymphocytes from SLE were similar to those from RSLE, but the changes were less obvious.

H₂S against proliferation

Kip1 and p21WAF1/CIP1

in lymphocytes of SLE patients.

H₂S changed the phosphorylation levels of AKT and GSK3β in lupus lymphocytes

Phosphorylation levels of AKT and its downstream target GSK3β increased in stimulated lupus lymphocytes, especially in those from RSLE, as what was demonstrated in our previously
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Table 1. Distribution of cells according to cell cycle phase. * p < 0.05 compared with Control with PHA treatment group. # p < 0.05 compared with SLE with PHA treatment group. Lymphocytes from healthy controls, SLE patients without renal involvement and RSLE patients were treated as indicated. -/-: untreated; -/+: treated with 2μg/ml PHA for 48 hours; +/+: treated with 4mM NaHS for 30 minutes before 2μg/ml PHA for 48 hours. N=5.

<table>
<thead>
<tr>
<th>Group</th>
<th>G0/G1</th>
<th>G1/S</th>
<th>S</th>
<th>G2/M</th>
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<tbody>
<tr>
<td>NaHS/PHA</td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>Control</td>
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<td>86.7±1.89</td>
<td>96.7±1.72</td>
<td>1.81±0.67</td>
</tr>
<tr>
<td>SLE</td>
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<td>77.2±5.12</td>
<td>96.3±2.1</td>
<td>2.11±0.71</td>
</tr>
<tr>
<td>RSLE</td>
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<td>68.4±1.81</td>
<td>97.3±5.14</td>
<td>2.04±0.59</td>
</tr>
</tbody>
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Fig. 4. H2S pretreatment changed cell cycle distribution of PHA-induced lymphocytes from SLE patients. Lymphocytes from healthy controls (A), SLE patients without renal involvement (B) and RSLE patients (C) were pretreated with or without NaHS (4mM) for 30 minutes in the presence or absence of PHA (2μg/ml) as indicated. 48 hours after different treatments, Cells were collected and analyzed by flow cytometry. NaHS/PHA: -/-: untreated; -/+: treated with PHA (2μg/ml) for 48 hours; +/+: treated with NaHS (4mM) for 30 minutes before PHA (2μg/ml) for 48 hours. N=5.

Fig. 5. The apoptosis percentages of lymphocytes from healthy controls, SLE and RSLE patients after treatment with or without NaHS at the indicated time points. Peripheral blood lymphocytes (PBL) were isolated from heparinized venous blood by Ficoll-Paque density gradient centrifugation. PBL were seeded at 1,000,000 cells per well in 24-well plates and treated with or without NaHS (4mM). Cells were collected at the indicated time points for apoptosis analysis using flow cytometry. It showed no obvious differences of apoptosis percentages of lymphocytes from healthy controls, SLE and RSLE patients between the groups treated with or without NaHS 4mM at 12, 24, 36 and 48 hours.

study [6]. As shown in Fig. 7A, B, C and D, the phosphorylation levels of AKT (ser473) and GSK3β (ser9) in lymphocytes of RSLE were increased significantly after stimulation with PHA. Pretreatment with NaHS in the absence of PHA attenuated the phosphorylation levels of AKT (ser473) but not of GSK3β (ser9). In the group receiving pretreatment with NaHS
and PHA stimulation, the phosphorylation levels of AKT (ser473) and GSK3β (ser9) were similar to the untreated group respectively. The expression of PTEN, which is the upstream regulator of AKT, was also influenced, decreasing in the PHA-induced group but increasing in the pretreatment with NaHS groups (either with or without PHA-induced). In order to further clarify the relation between H₂S and AKT/GSK3β signal pathway, the lymphocytes were stimulated with GSK3β inhibitor lithium chloride (LiCl, 12.5mM) for 48 hours in the absence or presence of NaHS pretreatment, and the cell viability was analyzed using CCK-8 assay. The increases of lymphocytes viability were 45.9% and 55.4% for SLE and RSLE lymphocytes respectively. Pretreatment with NaHS prior to exposure of lymphocytes to LiCl markedly inhibited the lymphocytes viability (Fig. 7E). These results suggested that AKT/GSK3β signal pathway was involved in the regulation of H₂S in the abnormal activation of lymphocytes from SLE patients.

Discussion

H₂S has been classified as a third neurotransmitter signaling molecule alongside nitric oxide (NO) and carbon monoxide (CO). Endogenous H₂S is produced by tissue-specific enzymes, including cystathionine-β-synthase (CBS), cystathionine-γ-lyase (CSE) and 3-mercaptosulfurtransferase (MST) [14]. So far, it is increasingly recognized as an important signaling molecule, regulating multiple cellular functions during normal and pathophysiological states. As a neurotransmitter, H₂S, which travels through the cell membranes without specific receptors, is involved in modulating cell proliferation and apoptosis in a variety of cells [7, 15]. NaHS is used as a common donor of H₂S. The concentration of NaHS used in the previous experiments were mainly at 20-1000 μM, rarely in 1-5 mM in the experiment studying rat intestinal crypt cells or human blood neutrophils [16]. Mirandola et al. showed that NaHS in 2 mM reduced the cellular cytotoxic response of peripheral blood lymphocytes [11], while recently W. Miller et al. showed that H₂S enhanced T cell activation in physiological levels (50–500 nM) [12]. In this study, we found that H₂S at
low concentrations slightly stimulated the viability of lupus lymphocytes, but inhibited the abnormal proliferation of lupus lymphocytes stimulated by PHA in higher concentration. The results were similar when using NaHS or GYY4137 as H₂S donor. The best inhibition effect achieved with pretreatment of NaHS at 4 mM group. Such discrepancies can be due to the different protocols using in experiments according to the different aims. NaHS was used after PHA-stimulation in the study of Mirandola et al, while NaHS treated cells before PHA-stimulation, using as a pretreated protection in this study. The various sources of experimental cells may be also responsible. In the studies of Mirandola et al, the peripheral blood lymphocytes were separated from healthy volunteers, while in W. Miller et al. cells were Jurkat T cells or from mice. However, it has been proved that the PHA-stimulated lymphocytes from SLE or RSLE proliferated more obviously than that from healthy controls in the present and previous studies [3, 6]. This is probably the reason why higher concentration of NaHS was needed to inhibit the PHA-stimulated proliferation of lymphocytes from lupus patients. Pretreatment with NaHS in 4 mM didn’t induce more apoptosis of lymphocytes from lupus patients than those from healthy controls (as shown in Fig. 5). However, the viability of lymphocytes was damaged when treated with NaHS at higher concentration in both healthy controls and lupus patients.

It has been established that H₂S acted as a cyclin dependent kinase inhibitor in cell cycle progression of the rat smooth muscle, human lung fibroblast, and oral epithelial cells and so on [7], and altered the expression of extracellular signal-regulated kinase (ERK),

**Fig. 7.** H₂S inhibited the abnormal proliferation of lymphocytes from RSLE through AKT/ GSK3β signal pathway. Lymphocytes separated from RSLE patients were treated with or without PHA for 48 hours in the presence or absence of NaHS at 4 mM for 30 min. Cell lysates were subjected to Western blot analysis using phospho-AKT(ser473), total AKT, phospho-GSK3β(ser9), total GSK3β, PTEN specific antibodies. A: representative figure. B, C, D: The mean± SD values of 5 individual samples are plotted (*: p<0.05, **: p<0.01 compared with NaHS/PHA -/- group). E: Lymphocytes separated from patients and controls were cultured for 48 hours with LiCl (12.5mM) with or without pretreatment of NaHS at 4 mM for 30 min. The viability of lymphocytes was measured by CCK-8. *: p<0.05.
p38 MAPK and p21 [17]. In this study, H₂S pretreatment (at the concentration of 4 mM) changed cell cycle distribution of PHA-stimulated lymphocytes from SLE and RSLE patients. NaHS pretreatment also modified the abnormal expressions of CDK2, p27kip1 and p21WAF1/CIP1 from lupus lymphocytes inducing by PHA-stimulation. The results were in accordance of the previous studies, which indicates that treatment of H₂S alters the abnormal expression of cell cycle regulatory proteins in lymphocytes from SLE and RSLE patients, and then prevents the entering of the S phase.

The AKT/GSK3β pathway is an important signal pathway in regulating the growth, proliferation and survival of lymphocytes. PTEN (phosphatase and tensin homologue) is a phosphoinositide 3-phosphatase that converts PtdIns (3,4,5)P3 back to PtdIns(4,5)P2. In cells lacking PTEN, basal PtdIns(3,4,5)P3 levels are increased and receptor stimulation causes exaggerated PI3K signaling [3]. Cristofano, A. et al. established that PTEN heterozygous (PTEN+/−) mice develop a lethal polyclonal autoimmune disorder, what’s more, T lymphocytes from these mice show reduced activation-induced cell death and increased proliferation upon activation [18]. AKT activation is mainly controlled by phosphorylation at Ser473 in human. Its downstream target, GSK3β activity is required to induce apoptosis of lymphocytes while its inhibitor promotes lymphocytes viability. The results of this study were consistent with these reports and our previously study [6]. H₂S in high concentration modified the abnormal activity of AKT/GSK3β signal pathway. Interestingly, pretreatment with H₂S alone suppressed the phosphorylation levels of AKT at ser473, but increased the expression of PTEN. It demonstrates that H₂S pretreatment induces the stable state of lupus lymphocytes through this signal pathway.

As the progression in the studies of H₂S, more and more therapeutic potential of H₂S have been found. Exogenous H₂S ameliorates myocardial dysfunction associated with the ischemia/reperfusion injury, and reduces the damage of gastric mucosa induced by anti-inflammatory drugs [8, 19]. But NaHS rapidly (within seconds) generated H₂S in buffer and did not affect plasma H₂S concentration when injected intravenously in animal experiments [20]. H₂S-donating drugs have been synthesized and tested in vivo and in vitro [21]. Chattopadhyay et al. showed that Hydrogen sulfide-releasing non-steroidal anti-inflammatory drugs (HS-NSAIDs) inhibited proliferation, induced apoptosis, and caused G0/G1 cell cycle block of HT-29 colon cancer cell [22]. John L. et al. established that HS-NSAIDs inhibited cyclooxygenase-1 and cyclooxygenase-2 activity as effectively as NSAIDs, and reduced the prostaglandin synthesis. What’s more, HS-NSAIDs did not induce leukocyte adherence whereas NSAIDs did [23]. These studies provide the clues for H₂S treatment to SLE, such as gastrointestinal protection, anti-inflammatory, vasodilatation and so on [24]. The results of this study provided evidence for therapeutic prospect of H₂S to SLE, suppressing the abnormal proliferation of lupus lymphocytes. Further studies are required, such as animal experiment in vivo and the effects of H₂S to different subsets of lymphocytes or organs from SLE and so on.

In conclusion, H₂S inhibited the abnormal activation and proliferation of lupus lymphocytes through AKT/GSK3β signal pathway.

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