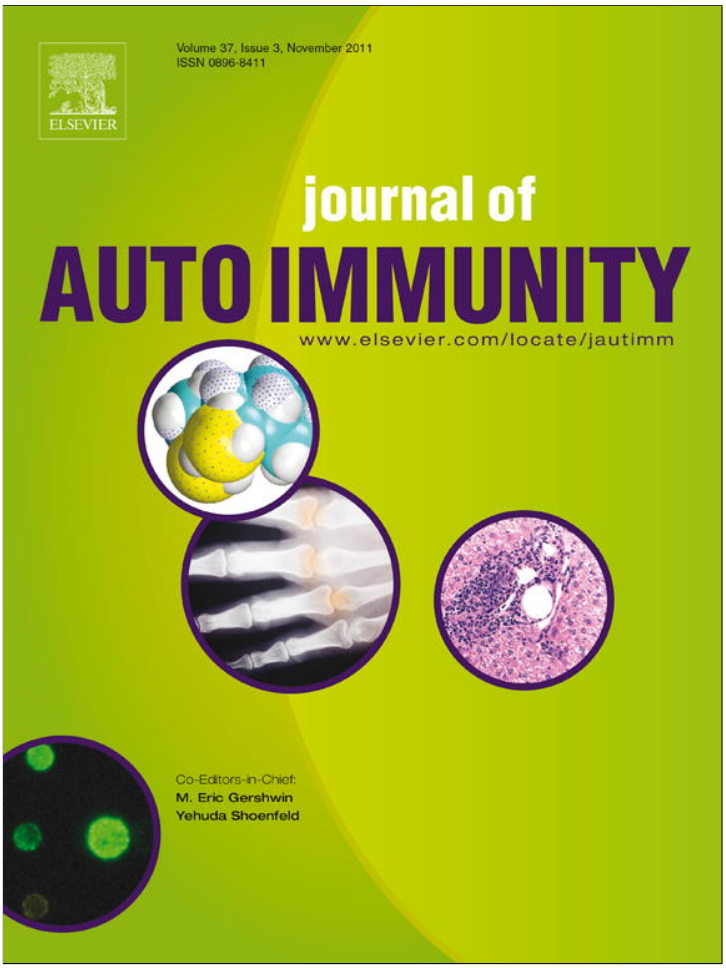

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Inhibited expression of hematopoietic progenitor kinase 1 associated with loss of jumonji domain containing 3 promoter binding contributes to autoimmunity in systemic lupus erythematosus

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ABSTRACT

Systemic lupus erythematosus (SLE) is an autoimmune disease characterized by T cell overactivation and B cell hyper-stimulation. Hematopoietic progenitor kinase 1 (HPK1, also called MAP4K1) negatively regulates T cell-mediated immune responses. However, the role of HPK1 and the mechanisms that regulate HPK1 expression in SLE remain poorly understood. Using chromatin immunoprecipitation (ChIP) microarray data, we identified markedly increased histone H3 lysine 27 trimethylation (H3K27me3) enrichment at the HPK1 promoter of SLE CD4+ T cells relative to controls, and confirmed this observation using ChIP and real-time PCR experiments. We further found that HPK1 mRNA and protein levels were significantly decreased in CD4+ T cells of patients with SLE, and that this decrease was not caused by exposure to standard SLE medications. Down-regulating HPK1 in healthy CD4+ T cells significantly accelerated T cell proliferation and production of IFNγ and IgG. Consistent with these findings, overexpressing HPK1 in SLE CD4+ T cells caused a significant decrease in T cell reactivity. In addition, we observed a striking decrease in jumonji domain containing 3 (JMJD3) binding, but no marked change in enhancer of zeste homolog 2 (EZH2) binding, at the HPK1 promoter region in SLE CD4+ T cells compared to healthy controls. SiRNA knock down of JMJD3 in healthy CD4+ T cells led to decreased JMJD3 binding and increased H3K27me3 enrichment at the HPK1 promoter region, thus inhibiting the expression of HPK1. Concordantly, plasmid-induced overexpression of JMJD3 in SLE CD4+ T cells led to increased JMJD3 binding, decreased H3K27me3 enrichment, and up-regulated HPK1 expression. Our results show for the first time that inhibited HPK1 expression in SLE CD4+ T cells is associated with loss of JMJD3 binding and increased H3K27me3 enrichment at the HPK1 promoter, contributing to T cell overactivation and B cell overstimulation in SLE. These findings suggest that HPK1 may serve as a novel target for effective SLE therapy.

1. Introduction

Autoimmune diseases are multifactorial in their pathogenesis. Contributory factors include: genetic predisposition and environmental factors [1,2]. Autoimmune diseases are always female-predominant [3–5]. As a typical chronic autoimmune disease, systemic lupus erythematosus (SLE) is characterized by T cell overactivation and the overproduction of autoantibodies against multiple self antigens [6]. Although the molecular mechanisms that initiate these autoimmune responses in SLE remain unclear, it is widely accepted that epigenetic alterations in the promoters of certain immune-related genes play critical roles in the onset and progression of SLE [7–10]. The term "epigenetics" refers to stable and heritable changes in gene expression that are not related to changes in the DNA sequence [11,12]. The major mechanisms of epigenetic regulation include DNA methylation, histone modifications, chromatin modifications, noncoding RNA regulation, and so forth [11–14]. Among histone modifications, histone H3 lysine 27 trimethylation (H3K27me3) is a hallmark of gene silencing [15–18], via its binding to Pc protein, which forms part of the polycomb repressive complex 1 (PRC1). PRC1 then blocks the access of transcriptional activation factors and chromatin remodeling factors to DNA, and prevents initiation of transcription by RNA polymerase II. Furthermore, PRC1 is known to associate with histone deacetylases

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(HDACs, which can inhibit transcription), and PRC1 and H3K27me3 can block positively-acting imprints such as H3K4 methylation [15,17]. It is well-known that the histone demethylase jumonji domain containing 3 (JMJD3) [19–22] and the histone methyltransferase enhancer of zeste homolog 2 (EZH2) [23,24] can both regulate H3K27me3 levels.

Hematopoietic progenitor kinase 1 (HPK1, also known as MAP4K1) is a mammalian Ste20-related serine/threonine protein kinase. It belongs to the germinal center kinase (GCK) family and can be activated by a variety of signal stimuli, such as epidermal growth factor (EGF) [25], prostaglandin E_2 (PGE_2) [26], transforming growth factor-β (TGF-β) [27], erythropoietin (EPO) [28], and TCR and BCR stimulation [29–32]. It is also involved in various cellular events, such as MAPK [33–36], NF-κB [29,37–39] and cytokine signalings [28,40,41], as well as cellular proliferation and apoptosis [31,42–44]. In addition, HPK1 negatively regulates TCR signaling and T cell-mediated immune responses [40,43]. Shui et al. found that $HPK1^{-/-}$ mice T cells become hyperproliferative in response to stimulation with anti-CD3 and anti-CD28 antibodies, and that these cells can produce more proinflammatory cytokines when immunized with T cell-dependent antigens. Furthermore, T cell-dependent humoral responses in $HPK1^{-/-}$ immunized mice are more vigorous than in controls, and $HPK1^{-/-}$ mice demonstrate even more severe autoimmune phenotypes in an experimental autoimmune encephalomyelitis (EAE) model [43]. The T cell phenotypes observed in these HPK1-deficient mice are reminiscent of those observed in the T cells of patients with SLE, suggesting that HPK1 may play a causative role in SLE pathogenesis.

In then present study, chromatin immunoprecipitation (ChIP) microarray analysis revealed that H3K27me3 enrichment at the HPK1 promoter was significantly higher in CD4+ T cells of patients with SLE than in healthy controls. According to this clue, we set out to further investigate the putative roles of HPK1 in the development of autoimmunity in SLE and epigenetic regulation of its expression. To achieve this, we first confirmed that HPK1 mRNA and protein expressions were significantly decreased in SLE CD4+ T cells. Secondly, we showed that HPK1 negatively regulated CD4+ T cell activation and production of IFNγ and IgG in both healthy and SLE CD4+ T cells. We also observed elevated H3K27me3 enrichment and decreased JMJD3 binding at the HPK1 promoter in SLE CD4+ T cells, with no change in EZH2 binding. By down- and up-regulating JMJD3 expression using JMJD3-siRNA and JMJD3-plasmid, respectively, we demonstrated that changes in HPK1 expression were associated with changes in JMJD3 binding and H3K27me3 enrichment at its promoter. Together, these results provide novel insights into the mechanisms that cause SLE, and suggest a new approach for the treatment of SLE.

2. Materials and methods

2.1. Subjects

15 patients with SLE and 15 age- and sex-matched healthy controls were enrolled in this study. Relevant patient information is listed in Table 1. All patients ($n = 15$, age: 28.60 ± 6.25 years) were recruited from the out-patient clinic and in-patient ward of the Department of Dermatology of the Second Xiangya Hospital, Central South University, China. All patients fulfilled at least 4 of the American College of Rheumatology (ACR) Classification Criteria for SLE [45]. Disease activity was assessed using the SLE Disease Activity Index (SLEDAI) [46]. Healthy donors ($n = 15$, age: 27.27 ± 6.42 years) were recruited from staff and graduate students at the Second Xiangya Hospital. Written informed consent was obtained from all subjects. This study was approved by the Human Ethics Committee of the Central South University Second Xiangya Hospital.

Table 1
Patient profiles.

Patient	Gender	Age	SLEDAI	Medications
1	Female	22	8	Pred[a] 15 mg/d
2	Female	21	4	Pred 10 mg/d
3	Female	31	15	None
4	Female	32	10	Pred 20 mg/d
5	Male	42	4	TG[b] 30 mg/d, HCQ[c] 0.2 g/d
6	Female	32	2	None
7	Female	35	2	Pred 5 mg/d
8	Female	30	18	None
9	Female	21	3	Pred 5 mg/d
10	Female	20	6	TG 30 mg/d, Pred 10 mg/d
11	Female	26	0	TG 30 mg/d
12	Female	31	20	Pred 20 mg/d
13	Female	28	0	None
14	Female	24	10	None
15	Female	34	14	Pred 15 mg/d

[a] Prednisone.
[b] Tripterygium glycoside.
[c] Hydroxychloroquine.

2.2. Cell isolation

Peripheral blood mononuclear cells (PBMCs) were isolated by Ficoll-Hypaque density gradient centrifugation (GE Healthcare). CD4+ T cells and CD19+ B cells were then isolated by positive selection using magnetic beads (Miltenyi), according to the protocol provided by the manufacturer. The purity of both enriched cell subsets was generally higher than 95%, as evaluated by flow cytometry.

2.3. ChIP microarrays

CD4+ T cells from 5 patients with SLE and 5 age- and sex-matched healthy controls were fixed with 1% formaldehyde for 10 min to cross-link histones to DNA, and then lysed with lysis buffer. Patient and healthy control lysates were pooled respectively and sent to Capitalbio (Beijing, China) for ChIP microarray analysis. ChIP quality control, labeling, hybridization, and scanning were performed by Capitalbio. Anti-H3K27me3 antibody-precipitated DNA and unprecipitated DNA (input) were labeled with Cy5 and Cy3, respectively. Samples were then cohybridized to the microarray panels, and Cy3/Cy5 ratio images of the microarrays were generated. In these images, diversified color intensities indicate relative H3K27me3 enrichment at various gene promoters. Statistical analyses were performed by Capitalbio. Compared to control CD4+ T cells, a relative increase or decrease in H3K27me3 promoter enrichment in SLE CD4+ T cells of at least two-fold was considered significant.

2.4. RNA extraction and real-time RT-PCR

Total RNA was isolated from CD4+ T cells with TRIzol Reagent (Invitrogen) according to the manufacturer's instructions, and stored at −80 °C. Real-time RT-PCR was performed with a Rotor-Gene3000 thermocycler (Corbett Research). mRNA levels were quantified using a SYBR PrimeScript RT-PCR kit (Takara). β-actin was also amplified as an endogenous control. A series of five dilutions from a RNA sample were included in order to generate a standard curve used to calculate the relative concentrations of transcript in every RNA sample. Negative controls (using water instead of RNA) were run for every experiment. All reactions were run in triplicate. Primers used were as follows: for HPK1, forward 5′-CTGCTGGAACGGAAAGAGAC-3′ and reverse 5′-CGGACAAGCAGG AATTTGTT-3′; for β-actin, forward 5′-CGCGAGAAGATGACCCAGAT-3′ and reverse 5′-GCACTGTGTTGGCGTACAGG-3′.

2.5. Western blotting

CD4+ T cells were lysed with whole cell lysis buffer and denatured at 100 °C for 5 min. Proteins were then separated by SDS-polyacrylamide gel electrophoresis, and transferred onto PVDF membranes (Millipore). The membranes were blocked in TBST buffer containing 5% non-fat dry milk, and incubated overnight at 4 °C with HPK1 antibody (1:100, Santa Cruz), JMJD3 antibody (1:100, Abgent), or β-actin antibody (1:1000, Santa Cruz). Experiments were repeated three times, and relative expression levels were quantified with Quantity One software (Bio-Rad).

2.6. Transfections

The PcDNA3.1 blank plasmid and pcDNA3.1-JMJD3-expressing plasmid were gifts from Dr. Charlie Degui Chen (Chinese Academy of Sciences). The PcDNA3 blank plasmid and pcDNA3-HPK1-expressing plasmid were gifts from Dr. Rüdiger Arnold (German Cancer Research Center). Control-siRNA, JMJD3-siRNA, and HPK1-siRNA were all designed and synthesized at Guangzhou RiboBio in China. Plasmid and siRNA transfections were performed using Human T Cell Nucleofector kits and a nucleofector (Amaxa), according to the manufacturer's instructions. Transfected cells were rested in human T cell culture medium containing 10% fetal bovine serum (FBS). CD4+ T cells transfected with JMJD3-siRNA, JMJD3 control-siRNA, pcDNA3.1-JMJD3-plasmid, or pcDNA3.1 blank plasmid were rested for 48 h in this medium before being subjected to further analysis. 5 h after transfection, CD4+ T cells transfected with HPK1-siRNA, HPK1 control-siRNA, pcDNA3-HPK1 plasmid, or pcDNA3 blank plasmid were incubated with 5.0 μg/ml anti-CD3 and 5.0 μg/ml anti-CD28 antibodies for the remaining 43 h, in order to activate HPK1.

2.7. Cell proliferation assays

5 h after transfection, 2×10^5 CD4+ T cells were seeded into every well of 96-well flat-bottomed plates, and stimulated with anti-CD3 and anti-CD28 antibodies for 43 h. 10 μl of 5 mg/ml MTT (Roche) was then added to each well. After 4 h, the plates were centrifuged and supernatants were removed. CD4+ T cells were subsequently dissolved in 100 μl DMSO with gentle shaking at room temperature. Absorbance at 570 nm was then measured using an ELISA reader (Bio-Tek ELx800 Absorbance Microplate Reader). Three replicate wells were used for every sample.

2.8. T-B cell cocultures for costimulation assays

Isolated B cells were cultured in RPMI 1640 medium with 10% FBS, 100 U/ml penicillin G, and streptomycin. After stimulation, CD4+ T cells were cocultured with autologous B cells at a ratio of 1:4 in 96-well round-bottomed plates for 8 d. Medium was supplemented on day 4, and supernatants were collected on day 8 to measure IgG concentrations.

2.9. ELISA

IFNγ concentrations in the supernatant of stimulated T cells were examined with an IFNγ quantification ELISA kit (Excell), and IgG concentrations in the supernatants of T-B cell cocultures were measured using an IgG quantification ELISA kit (Senxiong), both following the manufacturers' protocols. Three replicate wells were used for every sample, and all experiments were performed in triplicate. OD values were read at 450 nm for IFNγ examination and 490 nm for IgG quantification, using an ELISA reader (Bio-Tek ELx800 Absorbance Microplate Reader).

2.10. ChIP and real-time PCR

Chromatin immunoprecipitation (ChIP) assays were performed with a ChIP kit (Millipore), according to the manufacturer's protocol. In brief, CD4+ T cells were fixed with 1% formaldehyde for 10 min to cross-link histones to DNA, then lysed with lysis buffer. Cell lysates were sonicated to shear the DNA, and the sonicated extracts were then clarified by centrifugation. After preclearing with protein G-agarose beads, antibodies were added and incubated at 4 °C overnight on a 360° rotator. The next day, protein G-agarose beads were added and rotated for 1 h at 4 °C to collect immunoprecipitated complexes. The complexes were washed once with low-salt buffer, once with high-salt buffer, once with LiCl buffer, and twice with Tris–EDTA buffer, and then eluted with elution buffer. After reversing cross links between protein and DNA by heating at 65 °C for 4 h, the DNA was purified and subjected to real-time PCR analysis, with input DNA (total chromatin) as endogenous control. All experiments were performed in triplicate. The primers used to amplify the HPK1 promoter were: forward 5′-TTGTAGGGATGGGTTCTTGC-3′ and reverse 5′-ATCTTGGGGACTG CAAATGA-3′. The anti-H3K27me3 antibody was purchased from Millipore, the anti-JMJD3 antibody from Abgent, and the anti-EZH2 antibody from Abcam.

2.11. Statistical analysis

Results are expressed as mean ± SD. Variables were compared by Student's *t*-test (data from different transfections were compared by paired *t*-test, others by two-group *t*-test). Correlations were determined using Pearson's correlation coefficient. *P* values less than 0.05 were considered significant. All results were analyzed by SPSS 16.0 software (SPSS Inc.).

3. Results

3.1. Increased H3K27me3 enrichment at the HPK1 promoter in SLE CD4+ T cells in the result of ChIP microarrays

We first used ChIP microarray analysis to examine H3K27me3 enrichment at various gene promoters in pooled CD4+ T cell lysates from 5 SLE patients and 5 age- and sex-matched healthy controls. Based on the microarray results, out of 20,832 distinct gene promoters screened, 552 showed a greater than two-fold difference in H3K27me3 enrichment between SLE and control CD4+ T cells. Among these, H3K27me3 enrichment at the HPK1 promoter in SLE CD4+ T cells was approximately five times higher than in control CD4+ T cells (Fig. 1A and B).

3.2. Decreased HPK1 expression in CD4+ T cells of patients with SLE

We next compared HPK1 mRNA expression in SLE and control CD4+ T cells using real-time RT-PCR, and found that HPK1 mRNA levels were significantly decreased in SLE CD4+ T cells compared with healthy controls (Fig. 2A). The decreased expression of HPK1 in SLE CD4+ T cells was further confirmed at the protein level by western blotting (Fig. 2B and C). In addition, HPK1 mRNA levels were found to negatively correlate with disease activity as measured by SLEDAI (Fig. 2D).

Of the 15 patients with SLE, 10 had been treated with drugs including corticosteroids, antimalarials, and immunosuppressive agents (see Table 1). In order to investigate whether the changes in HPK1 expression observed in patients with SLE could be ascribed to drug treatment, we separately compared the 10 drug-treated patients and the 5 untreated patients with healthy controls. We found that both groups of SLE patients exhibited

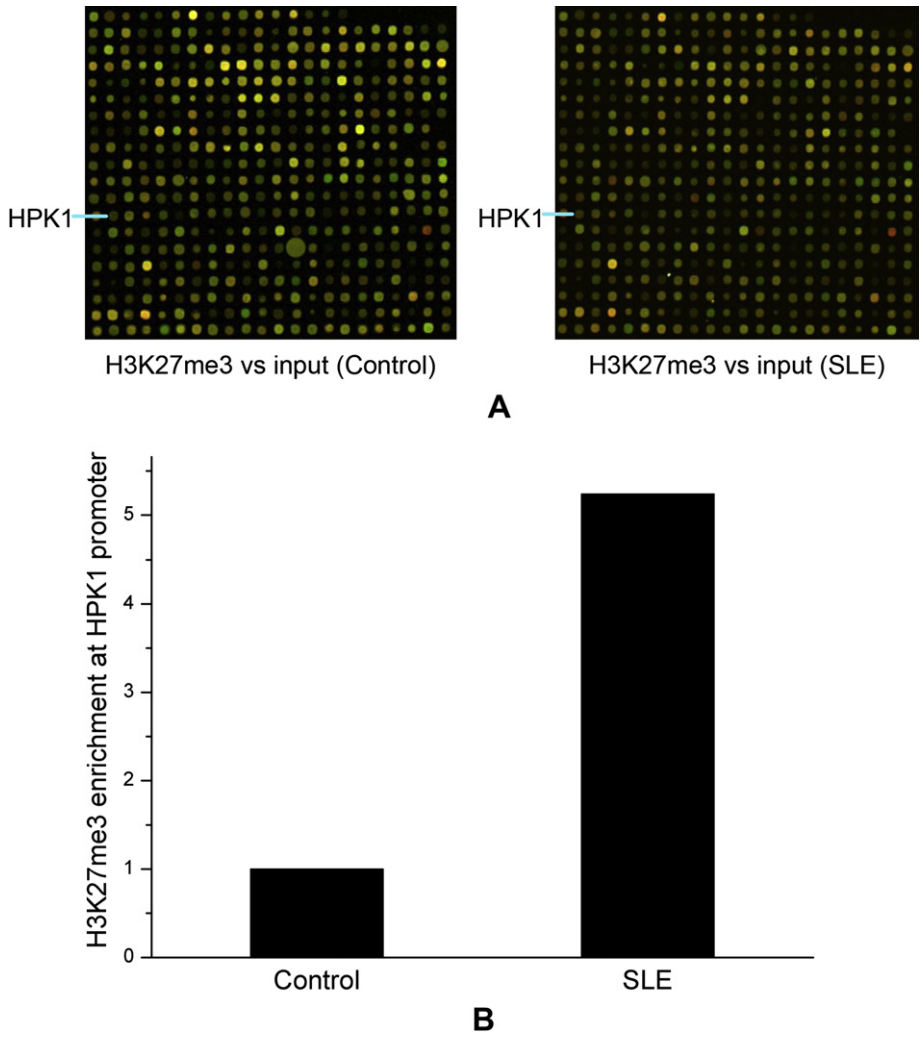

Fig. 1. ChIP microarray analysis of H3K27me3 enrichment in SLE and control CD4+ T cells. **A**. ChIP microarray panels showing relative H3K27me3 enrichment at various gene promoters in CD4+ T cell lysates pooled from 5 healthy controls (left-hand panel) and 5 patients with SLE (right-hand panel). Anti-H3K27me3 antibody-precipitated DNA and unprecipitated DNA (input) were respectively labeled with Cy5 (red) and Cy3 (green), and samples were subsequently cohybridized to microarray panels. Each individual dot shows the Cy3/Cy5 ratio representing relative H3K27me3 enrichment at a specific gene promoter. The HPK1 promoter dot (indicated by a blue line) is located in the second column, eight from the bottom. **B**. Relative H3K27me3 enrichment at the HPK1 promoter in SLE and healthy CD4+ T cells, quantified from the results shown in **A**. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

significantly decreased HPK1 mRNA levels when compared with healthy controls (Fig. 2E). We also compared HPK1 mRNA levels between treated patients and untreated SLE patients, and found that there was no significant difference between the two groups (Fig. 2E). These data suggest that altered HPK1 expression in SLE is unlikely to be caused by exposure to standard SLE medications.

3.3. Inhibiting HPK1 expression in healthy CD4+ T cells increases T cell reactivity

Subsequently, we determined the effects of HPK1 down-regulation on CD4+ T cell reactivity. CD4+ T cells from 3 healthy donors were isolated and transfected with HPK1-siRNA or control-siRNA. 5 h after transfection, they were stimulated with anti-CD3 and anti-CD28 antibodies for 43 h, after which supernatant IFNγ concentrations were measured by ELISA and CD4+ T cell proliferation was measured with an MTT assay. After stimulation, CD4+ T cells were cocultured with autologous B cells for 8 d, after which supernatants were collected to measure IgG concentrations. Compared to control-siRNA-transfected CD4+ T cells, we observed significantly decreased HPK1 protein levels (Fig. 3A and B) and greater cell proliferation (Fig. 3C) in cells transfected with HPK1-siRNA. Furthermore, supernatants collected from HPK1-siRNA-transfected CD4+ T cells showed higher levels of IFNγ than controls (Fig. 3D), and higher IgG supernatant concentrations in the B cell cocultures (Fig. 3E).

3.4. Overexpressing HPK1 in SLE CD4+ T cells inhibits T cell reactivity

We also transfected an HPK1 expression plasmid (pcDNA3-HPK1) or a control-plasmid (pcDNA3) into CD4+ T cells from 3 patients with SLE. As expected, marked overexpression of HPK1 was observed in HPK1 plasmid-transfected CD4+ T cells (Fig. 3F and G). Significantly inhibited T cell proliferation (Fig. 3H), decreased IFNγ secretion (Fig. 3I), and reduced IgG production (Fig. 3J) were all observed in SLE CD4+ T cells transfected with pcDNA3-HPK1, as compared to control-plasmid-transfected cells. These results indicate that HPK1 is a negative regulator of CD4+ T cell activation and reactivity, and that inhibition of HPK1 in SLE CD4+ T cells may lead to T cell overactivation and B cell hyper-stimulation.

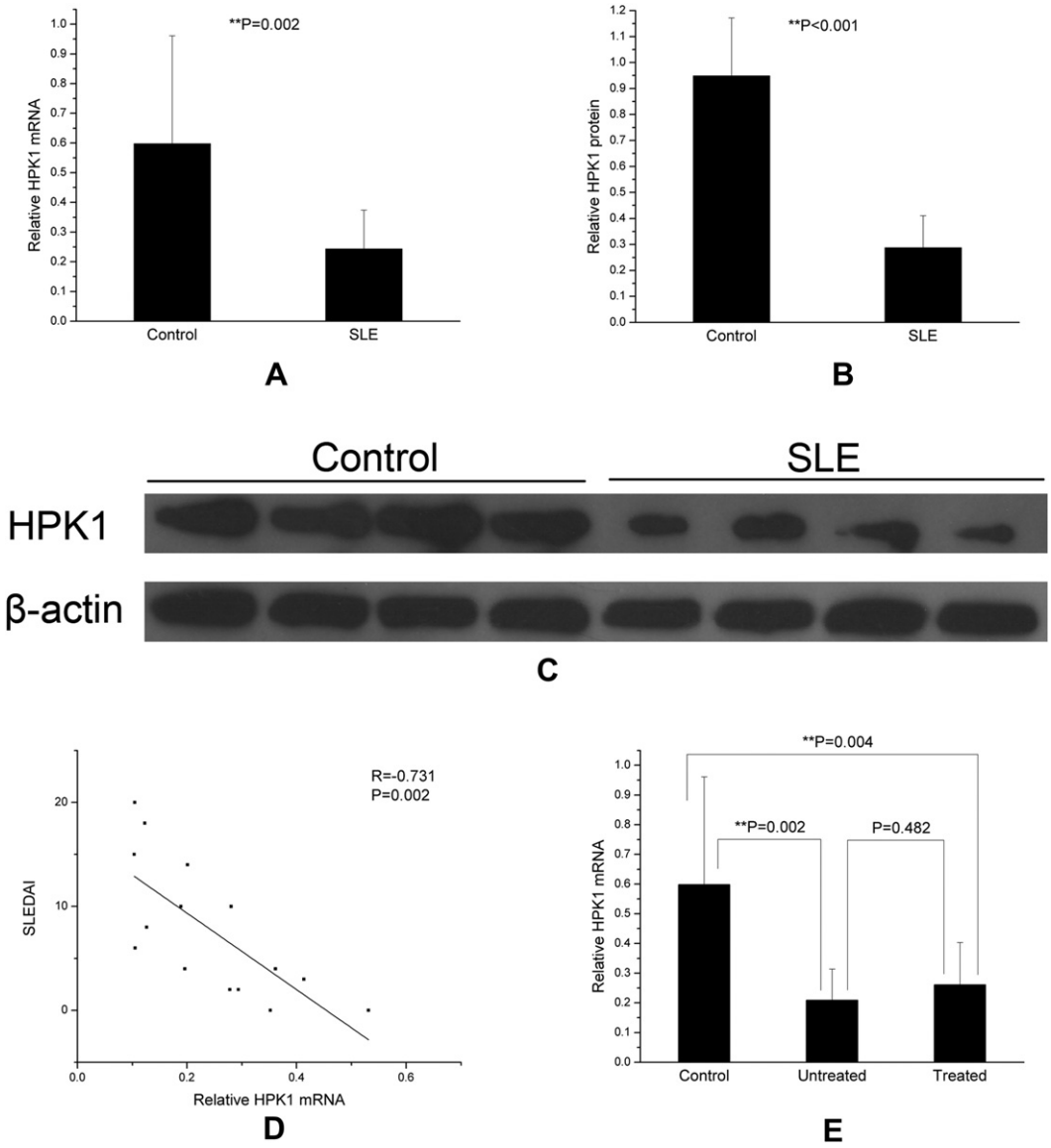

Fig. 2. HPK1 mRNA and protein expressions in SLE and healthy CD4+ T cells. **A**. Relative HPK1 mRNA levels in CD4+ T cells from 15 patients with SLE and 15 healthy controls were assessed by real-time RT-PCR and normalized to β-actin. **B** and **C**. HPK1 protein levels in CD4+ T cells from 15 patients with SLE and 15 healthy controls were measured by western blot analysis and normalized to β-actin. **D**. Negative correlation between HPK1 mRNA levels and SLEDAI score in CD4+ T cells of patients with SLE. **E**. Relative HPK1 mRNA levels in CD4+ T cells from 15 healthy controls, 10 drug-treated and 5 untreated patients with SLE were examined by real-time RT-PCR and normalized to β-actin. All data represent the mean of three independent experiments.

3.5. Increased H3K27me3 enrichment at the HPK1 promoter in SLE CD4+ T cells

To verify whether the epigenetic status of HPK1 was altered in SLE CD4+ T cells, we carried out ChIP and real-time PCR experiments to examine H3K27me3 enrichment at the HPK1 promoter of the 15 patients with SLE and 15 healthy controls. Compared to healthy controls, H3K27me3 enrichment at the HPK1 promoter was significantly increased in SLE CD4+ T cells (Fig. 4A), consistent with our previous ChIP microarray findings. Moreover, H3K27me3 enrichment at the promoter was negatively correlated with HPK1 mRNA levels in SLE CD4+ T cells (Fig. 4B).

3.6. Loss of JMJD3 binding at the HPK1 promoter in SLE CD4+ T cells

The increase in H3K27me3 levels in SLE CD4+ T cells prompted us to evaluate the status of two enzymes, JMJD3 and EZH2, which together modulate the methylation status of histone H3K27. ChIP followed by real-time PCR was performed to detect the levels of JMJD3 and EZH2 binding at the HPK1 promoter in CD4+ T cells from 15 patients with SLE and 15 healthy controls. A striking decrease was observed in JMJD3 binding at the HPK1 promoter in SLE CD4+ T cells compared with controls (Fig. 5A). In addition, the levels of JMJD3 binding were negatively correlated with H3K27me3 enrichment at the HPK1 promoter (Fig. 5B), and positively correlated with HPK1 mRNA levels (Fig. 5C). In contrast, EZH2 binding at the HPK1 promoter did not differ significantly between SLE and control groups (Fig. 5A).

3.7. JMJD3 down-regulation reduces HPK1 expression in healthy CD4+ T cells

In order to determine whether HPK1 down-regulation in SLE CD4+ T cells was due to loss of JMJD3 binding at the HPK1

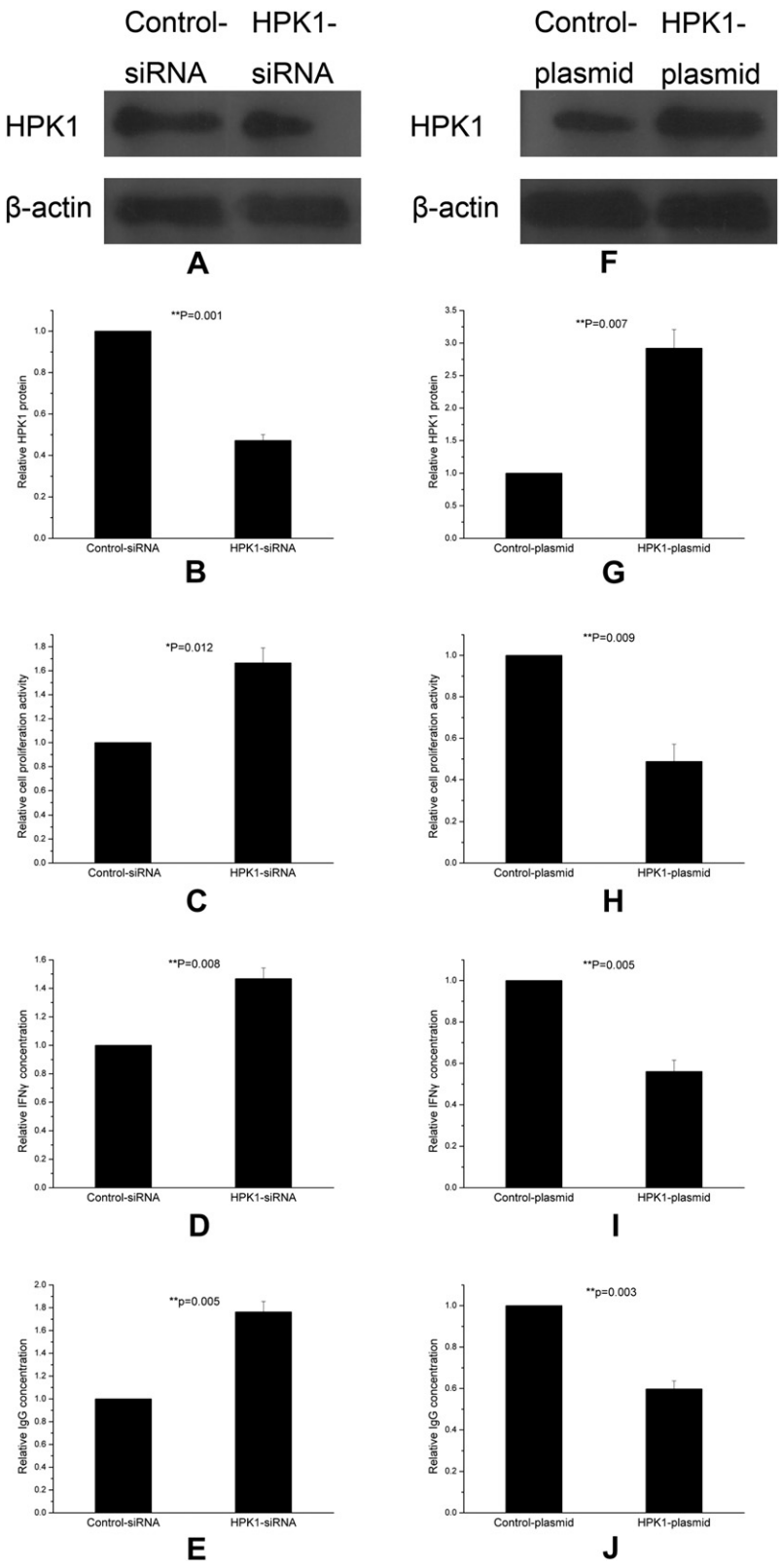

Fig. 3. Effects of HPK1 on CD4+ T cells from 3 healthy controls and 3 patients with SLE. **A**, **B, F**, and **G**. Relative HPK1 protein levels were assessed by western blot analysis of healthy CD4+ T cells (**A, B**) transfected with HPK1-siRNA or control-siRNA, and of SLE CD4+ T cells (**F, G**) transfected with HPK1-plasmid or control-plasmid. β-actin served as an endogenous control. Experiments were performed in triplicate. **C** and **H**. 5 h after transfection, partial cells were stimulated with 5.0 μg/ml anti-CD3 and 5.0 μg/ml anti-CD28 antibodies for 43 h, after which their proliferation rates were measured by MTT assay. Three replicate wells were used for every sample. **D** and **I**. After stimulation, IFNγ concentrations in the supernatant of CD4+ T cells were measured by ELISA. **E** and **J**. After stimulation, some CD4+ T cells were cocultured with autologous B cells for an additional 8 d, after which IgG concentrations in coculture supernatants were examined by ELISA. ELISA results represent the mean of three independent experiments, and three replicate wells were used for every sample.

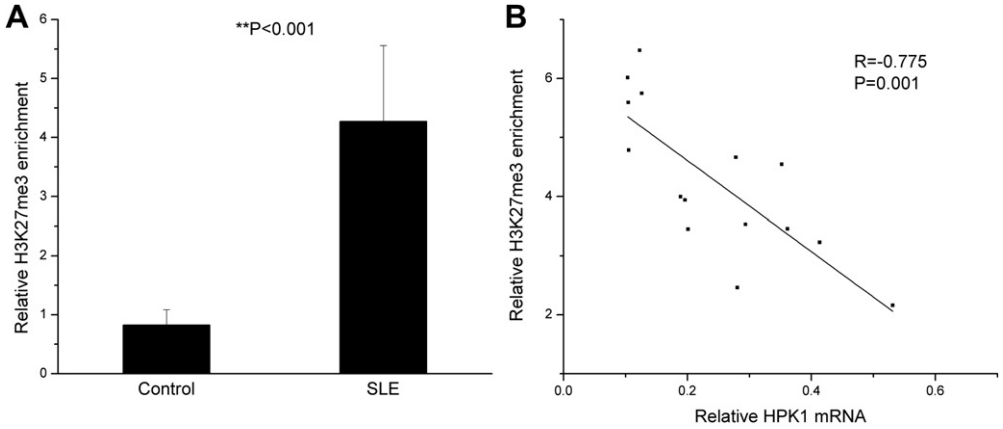

Fig. 4. H3K27me3 enrichment within the HPK1 promoter in CD4+ T cells from 15 patients with SLE and 15 healthy controls. **A**. Relative H3K27me3 levels within the HPK1 promoter in SLE and healthy CD4+ T cells were assessed by ChIP and real-time PCR. Results were normalized to input DNA (total chromatin). **B**. Negative correlation between the levels of H3K27me3 and HPK1 mRNA in SLE CD4+ T cells. All reactions were run in triplicate.

promoter, we transfected CD4+ T cells from 3 healthy donors with JMJD3-siRNA and evaluated the consequences on H3K27me3 and HPK1 expression. 48 h after transfection, JMJD3 expression was significantly inhibited by JMJD-siRNA compared to the control-siRNA group (Fig. 6A and B). JMJD3 binding at the HPK1 promoter was also decreased in the JMJD3-siRNA group (Fig. 6C). Concordantly, H3K27me3 enrichment at the HPK1 promoter was increased after JMJD3 down-regulation (Fig. 6D). As expected, western blot analysis showed that HPK1 expression was also markedly decreased in CD4+ T cells transfected with JMJD3-siRNA compared with controls (Fig. 6A and B).

3.8. Overexpressing JMJD3 in SLE CD4+ T cells restores HPK1 expression

To ascertain the effects of JMJD3 on HPK1 expression, we also transfected SLE CD4+ T cells with a JMJD3 expression plasmid (pcDNA3.1-JMJD3). 48 h after transfection, JMJD3 was significantly overexpressed in SLE CD4+ T cells compared to pcDNA3.1 blank plasmid-transfected controls (Fig. 6E and F). Concordant increases in JMJD3 binding at the HPK1 promoter were also detected (Fig. 6G), while H3K27me3 levels were decreased after JMJD3 overexpression (Fig. 6H). Interestingly, the repression of HPK1 expression in SLE CD4+ T cells was successfully reversed by transfection of the JMJD3 expression plasmid (Fig. 6E and F). These results indicate that JMJD3 can regulate HPK1 expression in SLE CD4+ T cells by modulating the status of H3K27me3 at the HPK1 promoter.

4. Discussion

In recent years, accumulating evidence has demonstrated that epigenetic alterations play essential roles in the pathogenesis of SLE [7–10]. In particular, considerable interest has been focused on DNA demethylation, and a growing number of studies indicate that demethylation of regulatory sequences can result in the overexpression of certain immunity-related genes, including perforin (PRF1) [47], CD11a (ITGAL) [48,49], CD70 (TNFSF7) [48,50], and CD40 ligand (CD40L) [51–53]. Up-regulation of these genes leads to T cell autoreactivity and immunoglobulin overproduction, ultimately contributing to the development of SLE. However, studies examining the role of histone modifications in SLE are limited.

H3K27me3 is known to inhibit gene transcription, and our ChIP microarray analysis showed that H3K27me3 enrichment at the HPK1 promoter of SLE CD4+ T cells was markedly higher than in healthy controls. We thus speculated that increased H3K27me3 levels may repress HPK1 expression in SLE CD4+ T cells. Since HPK1 is a negative regulator of TCR signaling and T cell-mediated immune responses [40,43], we hypothesized that the loss of HPK1 may play a causative role in the pathogenesis of SLE.

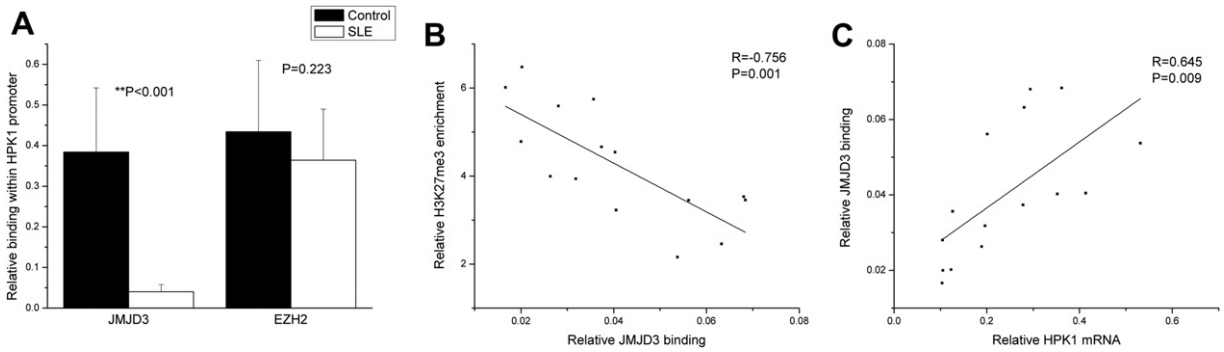

Fig. 5. Levels of JMJD3 and EZH2 binding within the HPK1 promoter region in CD4+ T cells from 15 patients with SLE and 15 healthy controls. **A**. Relative levels of JMJD3 and EZH2 protein within the HPK1 promoter region in SLE and healthy CD4+ T cells were assessed by ChIP and real-time PCR. Results were normalized to input DNA (total chromatin), and represent the mean of three independent experiments. **B**. Negative correlation between JMJD3 promoter binding and H3K27me3 levels in SLE CD4+ T cells. **C**. Positive correlation between JMJD3 promoter binding and HPK1 mRNA levels in SLE CD4+ T cells. All experiments were repeated three times.

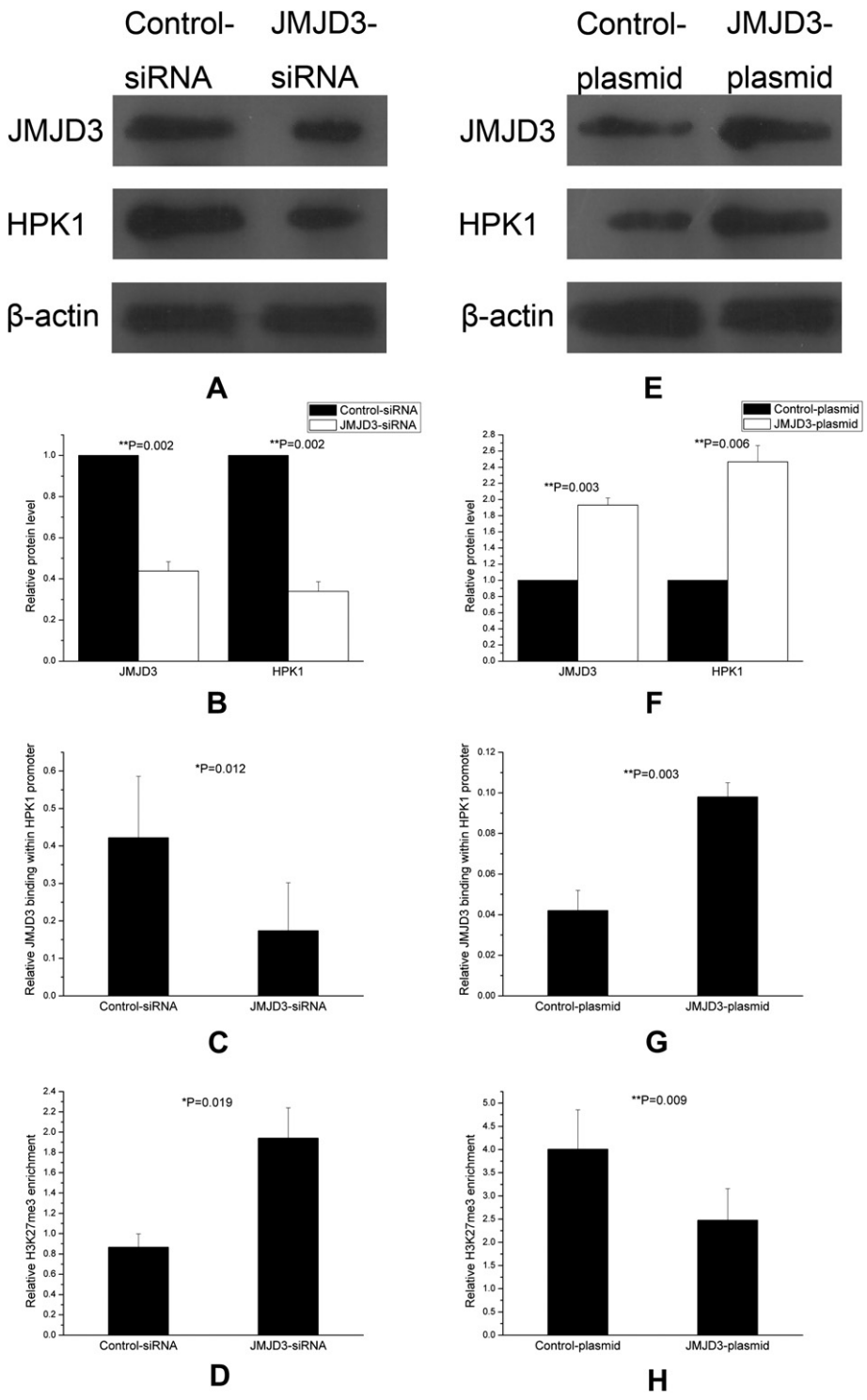

Fig. 6. Effects of JMJD3 up- and down-regulation on CD4+ T cells from 3 healthy controls and 3 patients with SLE. **A**, **B**, **E,** and **F**. Relative JMJD3 and HPK1 protein levels were assessed by western blot analysis of healthy CD4+ T cells (**A, B**) 48 h after transfection with JMJD3-siRNA or control-siRNA, and of SLE CD4+ T cells (**E, F**) 48 h after transfection with JMJD3-plasmid or control-plasmid. β-actin served as an endogenous control. **C** and **G**, Relative JMJD3 levels within the HPK1 promoter in healthy CD4+ T cells (**C**) transfected with JMJD3-siRNA or control-siRNA, and in SLE CD4+ T cells (**G**) transfected with JMJD3-plasmid or control-plasmid were confirmed by ChIP and real-time PCR 48 h after transfection. Results were normalized to input DNA (total chromatin). **D** and **H**, Relative H3K27me3 levels within the HPK1 promoter in healthy CD4+ T cells (**D**) transfected with JMJD3-siRNA or control-siRNA, and in SLE CD4+ T cells (**H**) transfected with JMJD3-plasmid or control-plasmid were confirmed by ChIP and real-time PCR 48 h after transfection. Results were normalized to input DNA (total chromatin). All experiments were performed in triplicate.

To understand the specific function of HPK1 in SLE CD4+ T cells and the mechanisms that regulate HPK1 expression, we first determined that HPK1 protein and mRNA levels were both significantly reduced in CD4+ T cells of patients with SLE. This difference is unlikely to be the result of medication use, because drug-treated and untreated SLE patients both exhibited significant decreases in HPK1 expression relative to healthy controls, and there was no significant difference between the drug-treated and untreated

groups. We also observed that HPK1 mRNA expression negatively correlated with disease activity in patients with SLE. Taken together, these data strongly suggest that HPK1 is involved in the development of SLE.

SLE is characterized by T cell overactivation and the overproduction of autoantibodies. The inflammatory cytokine IFNγ has also been found to play an important role in the development of SLE, and serum levels of IFNγ are increased in patients with SLE. Moreover, serum IFNγ levels correlate positively with SLE disease activity and levels of antibodies to DNA, and negatively with serum C3 levels [54,55]. Reducing the production of IFNγ can prolong survival, inhibit the onset of disease, and mitigate glomerulonephritis in lupus-prone mouse models [56,57]. To further characterize the role of HPK1 in SLE, we knocked down HPK1 expression in healthy CD4+ T cells using siRNA, and observed increased T cell proliferation, overproduction of IFNγ, and excessive B cell stimulation. On the other hand, plasmid-induced overexpression of HPK1 in SLE CD4+ T cells led to reduced T cell proliferative activity, and to decreased IFNγ and IgG synthesis. Therefore, we demonstrate for the first time that HPK1 plays important roles in SLE pathogenesis. These results agree with previously reported findings by Shui et al. [43], who investigated the role of HPK1 in mouse T cells, and with those of Alzabin et al. who demonstrated that HPK1$^{-/-}$ mice T cells can withstand PGE_2-mediated suppression of T cell proliferation and produce more IFNγ [40].

HPK1 can activate the α and β subunits of the IκB kinase (IKK) complex, resulting in IκB degradation and NF-κB activation [31,37,58]. During the proliferation phase that follows T cell activation, caspase-3 cleaves HPK1 into two fragments (the N-terminal region and the C-terminal region) [42,59,60], and the C-terminal domain becomes an inhibitor of NF-κB [44,59,60], a well-known pro-survival factor [37,61,62]. CD4+ T cells transfected with HPK1-siRNA not only lack full length HPK1, they also fail to produce the C-terminal region [58]. Thus, knocking down HPK1 relieves the negative regulation of NF-κB, which leads to CD4+ T cell hyperproliferation [63]. In contrast, overexpressing HPK1 decreases the proliferative activity of CD4+ T cells during the proliferation phase.

SLP-76 is a very important adaptor protein for HPK1. HPK1 is able to directly phosphorylate serine residues on SLP-76, consequently creating 14-3-3-binding sites and inducing the binding of 14-3-3ζ and τ isoforms to serine-phosphorylated SLP-76 [43,64]. The proteins 14-3-3ζ and τ are negative regulators of the TCR signaling pathway [43,64], and can bind and inhibit both PI3K and PKCθ. PI3K can trigger TCR-induced calcium signaling [43], and PKCθ is able to elevate the activity of Ras-related protein 1 (Rap1) [65]. Rap1 mediates LFA-1 integrin activation through its binding to RapL (a regulator for cell adhesion and polarization enriched in lymphoid tissues) and, as a result, assists T cells in adhering to ICAM-1 and spreading [65]. Moreover, upon TCR stimulation, adhesion and degranulation promoting adaptor protein (ADAP) binds SLP-76, which causes membrane recruitment of active Rap1. HPK1, which also binds SLP-76, will compete with ADAP for SLP-76 and reduce Rap1 recruitment [65]. In addition, HPK1 also has the potential to dampen Rap1 activation by sequestering C3G through the interaction of HPK1 with Crk-like protein (CrkL), which inhibits T cell activity [65]. Therefore, we speculate that decreased HPK1 expression is one of the reasons for CD4+ T cell overactivation in SLE.

Using real-time RT-PCR, we confirmed our ChIP microarray findings that H3K27me3 levels within the HPK1 promoter in SLE CD4+ T cells were significantly higher than in healthy controls. Furthermore, we detected a significant negative correlation between the levels of H3K27me3 and HPK1 mRNA. These findings suggest that increased H3K27me3 may be the main cause of HPK1 down-regulation in SLE CD4+ T cells. We also observed that JMJD3 binding at the HPK1 promoter was significantly reduced in SLE CD4+ T cells, and JMJD3 binding was negatively correlated with H3K27me3 levels, as well as positively correlated with HPK1 mRNA levels. On the other hand, there was no difference in EZH2 binding at the HPK1 promoter between patients with SLE and healthy controls. These data suggest that it is not an increase in EZH2, but a decrease in JMJD3 binding at the HPK1 promoter that leads to H3K27me3 up-regulation, which in turn inhibits the expression of HPK1. Moreover, we noticed that EZH2 binding at the HPK1 promoter was mildly decreased in patients with SLE, although this decrease was not significant.

Using siRNA-mediated knock down, we demonstrated that down-regulating JMJD3 in healthy CD4+ T cells reduced HPK1 levels and JMJD3 binding at the HPK1 promoter, while it increased H3K27me3 enrichment within the same region. In contrast, plasmid-induced overexpression of JMJD3 in SLE CD4+ T cells induced the opposite effects: an increase in HPK1 levels and JMJD3 promoter binding, with a corresponding decrease in H3K27me3 levels within the HPK1 promoter. Together, these data indicate that JMJD3 regulates the expression of HPK1, that this regulation is accomplished at least in part via the association of JMJD3 and H3K27me3 within the HPK1 promoter, and that the down-regulation in JMJD3 binding at the HPK1 promoter contributes to the reduction of HPK1 in SLE CD4+ T cells. Since our manipulations not only affected JMJD3 promoter binding, but also altered total JMJD3 levels, we cannot at this time eliminate the possibility that JMJD3 also regulates HPK1 in other ways.

In conclusion, the results of our study indicate that JMJD3 binding within the HPK1 promoter region is reduced in SLE CD4+ T cells, while H3K27me3 is enriched in the same area. These two factors inhibit HPK1 expression, leading to abnormal T cell reactivity and ultimately contributing to autoimmunity in SLE. Our findings also reveal that HPK1 is subjected to epigenetic regulation in SLE CD4+ T cells, and suggest that HPK1 may serve as an important target for effective SLE therapy.

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References

[1] Invernizzi P. Future directions in genetic for autoimmune diseases. J Autoimmun 2009;33:1–2.
[2] Youinou P, Pers JO, Gershwin ME, Shoenfeld Y. Geo-epidemiology and autoimmunity. J Autoimmun 2010;34:J163–7.
[3] Pessach IM, Notarangelo LD. X-linked primary immunodeficiencies as a bridge to better understanding X-chromosome related autoimmunity. J Autoimmun 2009;33:17–24.
[4] Larizza D, Calcaterra V, Martinetti M. Autoimmune stigmata in Turner syndrome: when lacks an X chromosome. J Autoimmun 2009;33:25–30.
[5] Persani L, Rossetti R, Cacciatore C, Bonomi M. Primary ovarian insufficiency: X chromosome defects and autoimmunity. J Autoimmun 2009;33:35–41.
[6] Zhao S, Long H, Lu Q. Epigenetic perspectives in systemic lupus erythematosus: pathogenesis, biomarkers, and therapeutic potentials. Clin Rev Allergy Immunol 2010;39:3–9.
[7] Hewagama A, Richardson B. The genetics and epigenetics of autoimmune diseases. J Autoimmun 2009;33:3–11.
[8] Pan Y, Sawalha AH. Epigenetic regulation and the pathogenesis of systemic lupus erythematosus. Transl Res 2009;153:4–10.
[9] Strickland FM, Richardson BC. Epigenetics in human autoimmunity. Epigenetics in autoimmunity – DNA methylation in systemic lupus erythematosus and beyond. Autoimmunity 2008;41:278–86.
[10] Cooper GS, Gilbert KM, Greidinger EL, James JA, Pfau JC, Reinlib L, et al. Recent advances and opportunities in research on lupus: environmental influences and mechanisms of disease. Environ Health Perspect 2008;116:695–702.

[11] Lu Q, Renaudineau Y, Cha S, Ilei G, Brooks WH, Selmi C, et al. Epigenetics in autoimmune disorders: highlights of the 10th Sjogren's syndrome symposium. Autoimmun Rev 2010;9:627–30.
[12] Brooks WH, Le Dantec C, Pers JO, Youinou P, Renaudineau Y. Epigenetics and autoimmunity. J Autoimmun 2010;34:J207–19.
[13] Sanchez-Pernaute O, Ospelt C, Neidhart M, Gay S. Epigenetic clues to rheumatoid arthritis. J Autoimmun 2008;30:12–20.
[14] Maciejewska-Rodrigues H, Karouzakis E, Strietholt S, Hemmatazad H, Neidhart M, Ospelt C, et al. Epigenetics and rheumatoid arthritis: the role of SENP1 in the regulation of MMP-1 expression. J Autoimmun 2010;35:15–22.
[15] Lund AH, van Lohuizen M. Polycomb complexes and silencing mechanisms. Curr Opin Cell Biol 2004;16:239–46.
[16] Cao R, Zhang Y. The functions of E(Z)/EZH2-mediated methylation of lysine 27 in histone H3. Curr Opin Genet Dev 2004;14:155–64.
[17] Kondo Y, Shen L, Cheng AS, Ahmed S, Boumber Y, Charo C, et al. Gene silencing in cancer by histone H3 lysine 27 trimethylation independent of promoter DNA methylation. Nat Genet 2008;40:741–50.
[18] Plath K, Fang J, Mlynarczyk-Evans SK, Cao R, Worringer KA, Wang H, et al. Role of histone H3 lysine 27 methylation in X inactivation. Science 2003;300:131–5.
[19] Hong S, Cho YW, Yu LR, Yu H, Veenstra TD, Ge K. Identification of JmjC domain-containing UTX and JMJD3 as histone H3 lysine 27 demethylases. Proc Natl Acad Sci U S A 2007;104:18439–44.
[20] Xiang Y, Zhu Z, Han G, Lin H, Xu L, Chen CD. JMJD3 is a histone H3K27 demethylase. Cell Res 2007;17:850–7.
[21] Swigut T, Wysocka J. H3K27 demethylases, at long last. Cell 2007;131:29–32.
[22] De Santa F, Totaro MG, Prosperini E, Notarbartolo S, Testa G, Natoli G. The histone H3 lysine-27 demethylase Jmjd3 links inflammation to inhibition of polycomb-mediated gene silencing. Cell 2007;130:1083–94.
[23] Kirmizis A, Bartley SM, Kuzmichev A, Margueron R, Reinberg D, Green R, et al. Silencing of human polycomb target genes is associated with methylation of histone H3 Lys 27. Genes Dev 2004;18:1592–605.
[24] Fujii S, Ito K, Ito Y, Ochiai A. Enhancer of zeste homologue 2 (EZH2) down-regulates RUNX3 by increasing histone H3 methylation. J Biol Chem 2008;283:17324–32.
[25] Ling P, Yao Z, Meyer CF, Wang XS, Oehrl W, Feller SM, et al. Interaction of hematopoietic progenitor kinase 1 with adapter proteins Crk and CrkL leads to synergistic activation of c-Jun N-terminal kinase. Mol Cell Biol 1999;19:1359–68.
[26] Sawasdikosol S, Russo KM, Burakoff SJ. Hematopoietic progenitor kinase 1 (HPK1) negatively regulates prostaglandin E2-induced fos gene transcription. Blood 2003;101:3687–9.
[27] Zhou G, Lee SC, Yao Z, Tan TH. Hematopoietic progenitor kinase 1 is a component of transforming growth factor beta-induced c-Jun N-terminal kinase signaling cascade. J Biol Chem 1999;274:13133–8.
[28] Nagata Y, Kiefer F, Watanabe T, Todokoro K. Activation of hematopoietic progenitor kinase-1 by erythropoietin. Blood 1999;93:3347–54.
[29] Zhou G, Boomer JS, Tan TH. Protein phosphatase 4 is a positive regulator of hematopoietic progenitor kinase 1. J Biol Chem 2004;279:49551–61.
[30] Arnold R, Frey CR, Muller W, Brenner D, Krammer PH, Kiefer F. Sustained JNK signaling by proteolytically processed HPK1 mediates IL-3 independent survival during monocytic differentiation. Cell Death Differ 2007;14:568–75.
[31] Brenner D, Golks A, Kiefer F, Krammer PH, Arnold R. Activation or suppression of NFkappaB by HPK1 determines sensitivity to activation-induced cell death. EMBO J 2005;24:4279–90.
[32] Sawasdikosol S, Pyarajan S, Alzabin S, Matejovic G, Burakoff SJ. Prostaglandin E2 activates HPK1 kinase activity via a PKA-dependent pathway. J Biol Chem 2007;282:34693–9.
[33] Siligardi G, Ruzza P, Hussain R, Cesaro L, Brunati AM, Pinna LA, et al. The SH3 domain of HS1 protein recognizes lysine-rich polyproline motifs. Amino Acids; 2011.
[34] Balakireva M, Rosse C, Langevin J, Chien YC, Gho M, Gonzy-Treboul G, et al. The Ral/exocyst effector complex counters c-Jun N-terminal kinase-dependent apoptosis in Drosophila melanogaster. Mol Cell Biol 2006;26:8953–63.
[35] Boomer JS, Tan TH. Functional interactions of HPK1 with adaptor proteins. J Cell Biochem 2005;95:34–44.
[36] Ito Y, Pandey P, Sathyanarayana P, Ling P, Rana A, Weichselbaum R, et al. Interaction of hematopoietic progenitor kinase 1 and c-Abl tyrosine kinase in response to genotoxic stress. J Biol Chem 2001;276:18130–8.
[37] Hu MC, Wang Y, Qiu WR, Mikhail A, Meyer CF, Tan TH. Hematopoietic progenitor kinase-1 (HPK1) stress response signaling pathway activates IkappaB kinases (IKK-alpha/beta) and IKK-beta is a developmentally regulated protein kinase. Oncogene 1999;18:5514–24.
[38] Lewitzky M, Harkiolaki M, Domart MC, Jones EY, Feller SM. Mona/Gads SH3C binding to hematopoietic progenitor kinase 1 (HPK1) combines an atypical SH3 binding motif, R/KXXK, with a classical PXXP motif embedded in a polyproline type II (PPII) helix. J Biol Chem 2004;279:28724–32.
[39] Yu J, Riou C, Davidson D, Minhas R, Robson JD, Julius M, et al. Synergistic regulation of immunoreceptor signaling by SLP-76-related adaptor Clnk and serine/threonine protein kinase HPK-1. Mol Cell Biol 2001;21:6102–12.
[40] Alzabin S, Pyarajan S, Yee H, Kiefer F, Suzuki A, Burakoff S, et al. Hematopoietic progenitor kinase 1 is a critical component of prostaglandin E2-mediated suppression of the anti-tumor immune response. Cancer Immunol Immunother 2010;59:419–29.
[41] Konigsberger S, Peckl-Schmid D, Zaborsky N, Patzak I, Kiefer F, Achatz G. HPK1 associates with SKAP-HOM to negatively regulate Rap1-mediated B-lymphocyte adhesion. PLoS One 2010;5.
[42] Brenner D, Golks A, Becker M, Muller W, Frey CR, Novak R, et al. Caspase-cleaved HPK1 induces CD95L-independent activation-induced cell death in T and B lymphocytes. Blood 2007;110:3968–77.
[43] Shui JW, Boomer JS, Han J, Xu J, Dement GA, Zhou G, et al. Hematopoietic progenitor kinase 1 negatively regulates T cell receptor signaling and T cell-mediated immune responses. Nat Immunol 2007;8:84–91.
[44] Schulze-Luehrmann J, Santner-Nanan B, Jha MK, Schimpl A, Avots A, Serfling E. Hematopoietic progenitor kinase 1 supports apoptosis of T lymphocytes. Blood 2002;100:954–60.
[45] Tan EM, Cohen AS, Fries JF, Masi AT, McShane DJ, Rothfield NF, et al. The 1982 revised criteria for the classification of systemic lupus erythematosus. Arthritis Rheum 1982;25:1271–7.
[46] Bombardier C, Gladman DD, Urowitz MB, Caron D, Chang CH. Derivation of the SLEDAI. A disease activity index for lupus patients. The Committee on Prognosis Studies in SLE. Arthritis Rheum 1992;35:630–40.
[47] Lu Q, Wu A, Ray D, Deng C, Attwood J, Hanash S, et al. DNA methylation and chromatin structure regulate T cell perforin gene expression. J Immunol 2003;170:5124–32.
[48] Zhao M, Sun Y, Gao F, Wu X, Tang J, Yin H, et al. Epigenetics and SLE: RFX1 downregulation causes CD11a and CD70 overexpression by altering epigenetic modifications in lupus CD4+ T cells. J Autoimmun 2010;35:58–69.
[49] Lu Q, Ray D, Gutsch D, Richardson B. Effect of DNA methylation and chromatin structure on ITGAL expression. Blood 2002;99:4503–8.
[50] Lu Q, Wu A, Richardson BC. Demethylation of the same promoter sequence increases CD70 expression in lupus T cells and T cells treated with lupus-inducing drugs. J Immunol 2005;174:6212–9.
[51] Sawalha AH, Harley JB, Scofield RH. Autoimmunity and Klinefelter's syndrome: when men have two X chromosomes. J Autoimmun 2009;33:31–4.
[52] Invernizzi P, Pasini S, Selmi C, Gershwin ME, Podda M. Female predominance and X chromosome defects in autoimmune diseases. J Autoimmun 2009;33:12–6.
[53] Lu Q, Wu A, Tesmer L, Ray D, Yousif N, Richardson B. Demethylation of CD40LG on the inactive X in T cells from women with lupus. J Immunol 2007;179:6352–8.
[54] Hayashi T. Therapeutic strategies for SLE involving cytokines: mechanism-oriented therapies especially IFN-gamma targeting gene therapy. J Biomed Biotechnol; 2010.
[55] Funauchi M, Sugishima H, Minoda M, Horiuchi A. Serum level of interferon-gamma in autoimmune diseases. Tohoku J Exp Med 1991;164:259–67.
[56] Dong L, Ito S, Ishii KJ, Klinman DM. Suppressive oligonucleotides protect against collagen-induced arthritis in mice. Arthritis Rheum 2004;50:1686–9.
[57] Gerez L, Shkolnik T, Hirschmann O, Lorber M, Arad G, Kaempfer R. Hyper-inducible expression of the interferon-gamma (IFN-gamma) gene and its suppression in systemic lupus erythematosus (SLE). Clin Exp Immunol 1997;109:296–303.
[58] Brenner D, Brechmann M, Rohling S, Tapernoux M, Mock T, Winter D, et al. Phosphorylation of CARMA1 by HPK1 is critical for NF-kappaB activation in T cells. Proc Natl Acad Sci U S A 2009;106:14508–13.
[59] Chen YR, Meyer CF, Ahmed B, Yao Z, Tan TH. Caspase-mediated cleavage and functional changes of hematopoietic progenitor kinase 1 (HPK1). Oncogene 1999;18:7370–7.
[60] Arnold R, Liou J, Drexler HC, Weiss A, Kiefer F. Caspase-mediated cleavage of hematopoietic progenitor kinase 1 (HPK1) converts an activator of NFkappaB into an inhibitor of NFkappaB. J Biol Chem 2001;276:14675–84.
[61] Brenner D, Krammer PH, Arnold R. Concepts of activated T cell death. Crit Rev Oncol Hematol 2008;66:52–64.
[62] Krammer PH, Arnold R, Lavrik IN. Life and death in peripheral T cells. Nat Rev Immunol 2007;7:532–42.
[63] Acuto O, Di Bartolo V, Michel F. Tailoring T-cell receptor signals by proximal negative feedback mechanisms. Nat Rev Immunol 2008;8:699–712.
[64] Di Bartolo V, Montagne B, Salek M, Jungwirth B, Carrette F, Fourtane J, et al. A novel pathway down-modulating T cell activation involves HPK-1-dependent recruitment of 14-3-3 proteins on SLP-76. J Exp Med 2007;204:681–91.
[65] Patzak IM, Konigsberger S, Suzuki A, Mak TW, Kiefer F. HPK1 competes with ADAP for SLP-76 binding and via Rap1 negatively affects T-cell adhesion. Eur J Immunol 2010;40:3220–5.