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EZH2 modulates the DNA methylome and controls T cell adhesion through junctional adhesion molecule-A in lupus patients

Pei-Suen Tsou, PhD[1], Patrick Coit, MPH[1], Nathan C. Kilian, BSc[1], and Amr H. Sawalha, MD[1,2]

[1]Division of Rheumatology, Department of Internal Medicine, University of Michigan, Ann Arbor, MI

[2]Center for Computational Medicine and Bioinformatics, University of Michigan, Ann Arbor, MI

Address correspondence: Amr H. Sawalha, Division of Rheumatology, University of Michigan, 1150 W Medical Center Dr. 5520 MSRB1, SPC 5680, Ann Arbor, MI 48109, USA. Telephone (734) 763-1858. Fax (734) 763-4151. Email: asawalha@umich.edu.

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Abstract

Objective: EZH2 is an epigenetic regulator that mediates H3K27 trimethylation and modulates DNA methylation. The aim of this study is to characterize the role of EZH2 in CD4+ T cells upon lupus pathogenesis.

Methods: EZH2 expression levels were determined in CD4+ T cells isolated from lupus patients and healthy controls. The epigenetic effects of EZH2 overexpression in CD4+ T cells were evaluated using a genome-wide DNA methylation approach. Gene expression and miRNAs were assessed by qPCR while protein expression was examined by Western blotting. A cell adhesion assay was used to assess adhesion of CD4+ T cells to human microvascular endothelial cells.

Results: EZH2 and H3K27me3 levels were increased in CD4+ T cells in lupus compared to healthy controls. MiR-26a and miR-101 downregulated EZH2, and were reduced in lupus CD4+ T cells. Overexpressing EZH2 in CD4+ T cells resulted in significant DNA methylation changes. Genes involved in leukocyte adhesion and migration, including *F11R* encoding JAM-A, become hypomethylated in CD4+ T cells when EZH2 is overexpressed. Overexpression of EZH2 resulted in increased JAM-A expression and CD4+ T cell adhesion. Pre-incubation of EZH2-transfected CD4+ T cells with neutralizing antibodies against JAM-A significantly blunted cell adhesion. Similarly,

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CD4+ T cells from lupus patients overexpressed JAM-A and adhered significantly more to endothelial cells compared to T cells from healthy controls. Blocking JAM-A or EZH2 significantly reduced endothelial cell adhesion of lupus CD4+ T cells.

Conclusions: We identified a novel role for EZH2 in T cell adhesion mediated by epigenetic remodeling and upregulation of JAM-A. Blocking EZH2 or JAM-A might have a therapeutic potential in lupus by reducing T cell adhesion, migration, and extravasation.

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Introduction

Systemic lupus erythematosus is a chronic relapsing autoimmune disease characterized by autoantibody production and a wide variety of clinical manifestations with multiple organ involvement. Although the underlying cause for lupus is largely unknown, substantial research has pointed to the involvement of both genetic and epigenetic mechanisms (1, 2). In particular, DNA methylation, an epigenetic mark that is heritable, stable, and reversible, has been shown to play crucial roles in mediating the pathogenesis of lupus (3, 4). It not only affects gene expression patterns and function in lupus CD4+ T cells (5, 6), but also primes interferon-regulated genes in lupus naïve CD4+ T cells to be epigenetically poised to respond to interferon upon activation (4). CD4+ T cells from lupus patients with active disease show more pronounced DNA methylation defect than those with inactive disease (7-9).

In a recent study, we suggested that with lupus flares, naïve CD4+ T cells undergo a shift in DNA methylation to favor T cell activation and poise CD4+ T cells for Th2, Th17, and follicular helper T cell responses (10). Through bioinformatics and literature mining analyses, a significant enrichment of EZH2 binding sites was found in loci that showed progressive increase in DNA methylation levels with lupus disease activity, suggesting a role for EZH2 in lupus progression. In addition, the two miRNAs, miR-101 and miR-26a, that target and regulate EZH2, were negatively correlated with disease activity in naïve CD4+ T cells from lupus patients.

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In this study, we set forth to follow up on the role of EZH2 in lupus naïve CD4+ T cells. We sought to determine the expression of EZH2 along with the two miRNAs that control EZH2 expression in naïve CD4+ T cells from control and lupus patients. Through examination of changes in genome-wide DNA methylation status after overexpression of EZH2 in naïve CD4+ T cells, we discovered a novel functional role for EZH2 in CD4+ T cells with potential therapeutic implication in lupus.

Materials and Methods

Patients and controls. All patients included in our study met the American College of Rheumatology criteria for the classification of lupus (11). Both patients (57 females; median age = 42, range 19–60, mean age ± SD = 40 ± 11) and healthy controls (40 females; median age = 44, range 20–63, mean age ± SD = 44 ± 11) were recruited from the University of Michigan Rheumatology Clinics. Systemic lupus erythematosus disease activity index (SLEDAI) scores of the patients ranged from 0 to 12, with a mean of 3 and a median of 2. All subjects included in this study signed a written informed consent. All procedures in this study were reviewed and approved by the Institutional Review Board of the University of Michigan.

Cell isolation and culture. Naïve CD4+ T cells were isolated from whole blood using a naïve CD4+ T cell isolation kit (Miltenyi Biotec) which allows for the indirect isolation of untouched naïve CD4+ T cells as previously described (10). Where indicated, isolated naïve CD4+ T cells were stimulated with anti-CD3 (10 μg/ml, pre-coated on plate) and anti-CD28 (2.5 μg/ml) overnight. Human microvascular endothelial cells (HMVECs) were grown in EBM-2 media with growth factors (Lonza). Cells between passage 3 and 9 were used in the experiments.

Overexpression experiments. Overexpression of *EZH2* in naïve CD4+ T cells was performed using the Amaxa 4D-Nucleofactor System (Lonza). After isolation and purification, naïve CD4+ T cells from healthy subjects were transfected with 0.1 μg of *EZH2* (Origene; control vector pCMV6-XL5) and cultured in RPMI media supplemented with 10% fetal bovine serum (FBS) and 2mM L-glutamine. After 5 hours of transfection,

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culture media were changed to remove the transfection reagent and the cells were stimulated with anti-CD3 and anti-CD28 overnight. The cells were cultured for an additional 48 hours before protein and RNA were collected. DNA was also extracted for the DNA methylation assessment described below. Similar procedures were carried out for miRNA overexpression experiments using the Amaxa 4D-Nucleofactor System. Naïve CD4+ T cells from healthy subjects were transfected with 500 nM of miR-26a or miR-101 (mirVana® miRNA mimic, ThermoFisher Scientific) and stimulated overnight. RNA was collected at day 3 post-transfection. Cell survival rate for the miRNA transfected cells was approximately 55%.

mRNA extraction and qRT-PCR. Total RNA from cells was isolated using Direct-zol™ RNA MiniPrep Kit (Zymo Research). Preparation of cDNA was done using the Verso cDNA synthesis kit (ThermoFisher Scientific). Primers for human *EZH2*, *DNMT1*, *DNMT3A*, *MECP2* and *β-actin* along with Power SYBR Green PCR master mix (Applied Biosystems) were used for qPCR, which was run by a ViiA™ 7 Real-Time PCR System. Primer sequences are as follows: *DNMT1* FW: CGACTACATCAAAGGCAGCAACCTG; RV: TGGAGTGGACTTGTGGGTGTTCTC; *DNMT3A*: FW: CGAGTCCAACCCTGTGATGATTG; RV: CGTGGTCTTTGCCCTGCTTTATG; *β-actin* FW: GTCAGGCAGCTCGTAGCTCT; RV: GCCATGTACGTTGCTATCCA. The *EZH2* primers were KiCqStart® SYBR® Green Primers from Sigma and the *MECP2* primers were purchased from Qiagen (QuantiTect Primer Assays). MiRNAs were analyzed using the TaqMan Advanced miRNA assays from Thermo Fisher Scientific.

Western blots. Cell lysate was prepared from stimulated CD4+ T cells from both healthy subjects and lupus patients. Proteins were separated by SDS-PAGE and electroblotted onto nitrocellulose membranes. EZH2, junctional adhesion molecule-A (JAM-A), and H3K27me3 were detected using anti-human EZH2 antibodies (Cell Signaling), anti-JAM-A antibodies (Santa Cruz Biotechnology), and anti-H3K27me3 antibodies (Cell Signaling), while β-actin and histone H3 were used as a loading control (anti-β-actin antibodies were from Sigma Aldrich; anti-H3 antibodies were from Cell Signaling). Images were visualized by Omega Lum C Imaging System (Gel Company) and quantification of the bands was performed using GelQuant.NET (BiochemLab Solutions).

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DNA methylation assessment and analysis. Genomic DNA, which was isolated from control and EZH2-overexpressing naïve CD4+ T cells from 4 healthy subjects with and without stimulation, was bisulfite converted using an EZ DNA Methylation kit (Zymo Research). Genome-wide DNA methylation status in these samples was then evaluated using the Illumina Infinium Methylation EPIC BeadChip Array. The Illumina GenomeStudio platform was used to analyze the methylation data as previously described (4). The average level of DNA methylation (β) on each CpG site was compared between control and EZH2-overexpressing samples. Differentially methylated CpG sites were defined as those with a differential methylation score ≥|22| (equivalent to p value of less than 0.05 after adjusting for multiple testing) and a mean methylation difference greater than 10% between the 2 groups. Differentially methylated genes were analyzed for Gene Ontology (GO), network, and pathway enrichments using the Database for Annotation, Visualization and Integrated Discovery (DAVID V.6.7) (12, 13).

***In vitro* cell adhesion assay.** An adhesion assay of the naïve CD4+ T cells to HMVECs was carried out as previously described with slight modification (14). HMVECs were grown in 96-well plates and stimulated with TNFα (25 ng/ml) in EBM-2 media containing 0.1% FBS. Overexpression of EZH2 in naïve CD4+ T cells was carried out as described above. Stimulated EZH2-overexpressing CD4+ T cells (50,000 cells/well in 100 μl RPMI medium) were labeled with Calcein AM (cell-permeant dye, 5 μM; Invitrogen) in the presence or absence of neutralizing antibodies to JAM-A (50 μg/ml, BioLegend) or isotype control (Mouse IgG1, κ, BioLegend). The fluorescent-tagged T cells were washed with PBS twice and then added to HMVECs and incubated for 30 minutes. Non-adherent T cells were washed off, and fluorescence was measured at 485/528 nm using a Synergy HT fluorescence plate reader (BioTek Instruments). We also examined adhesion of lupus CD4+ T cells to HMVECs. Naïve CD4+ T cells were isolated from lupus patients and stimulated overnight. Cells were cultured for an additional 2 days before they were assayed for adhesion as described above. The effect of JAM-A for lupus T cells was also evaluated using neutralizing antibodies to JAM-A. After culturing for 3 days, stimulated CD4+ T cells from lupus patients were washed in PBS and labeled with Calcein AM in the presence or absence of neutralizing antibodies to JAM-A or isotype control. After washing with PBS the fluorescent-tagged T

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cells were subjected to adhesion assays described above. To determine whether inhibition of EZH2 affected T cell adhesion, naïve CD4+ T cells from lupus patients were stimulated overnight in the presence of DZNep (5 μM, Cayman Chemical, dissolved in PBS), and media was changed to remove the stimulants the next day. T cells were treated with DZNep for additional 2 days and the cells were washed with PBS twice then assayed for adhesion. Cell survival rate for DZNep-treated cells was approximately 77%. Meanwhile, an aliquot of cells was lysed and saved for Western blotting to check the expression of EZH2, JAM-A, and H3K27me3 after DZNep treatment. To visualize T cell adhesion to HMVECs, dye-tagged stimulated CD4+ T cells from healthy controls, lupus patients, and lupus patients treated with DZNep were plated on HMVECs that were labeled with the red fluorescence dye PKH26 (Sigma). Pictures were taken using the EVOS FL Cell Imaging System (Thermo Fisher). The effect of GSK126, which inhibits the catalytic activity but not expression of EZH2 (15), was also used to evaluate the effect of EZH2 on cell adhesion. Procedures were similar to what was done with DZNep. The final concentration of GSK126 (Cayman Chemical) was 5 μM and DMSO was used as the vehicle control.

Statistical analysis. Results were expressed as mean ± S.D. To determine the differences between the groups, Mann-Whitney U test was performed using GraphPad Prism version 6 (GraphPad Software, Inc). One-way ANOVA with post-hoc test (Sidak) was used to analyze Figure 2A-C as well as Figure 3B and 3D. Two-way ANOVA with post-hoc test (Tukey) was used to identify differences in EZH2 in control and lupus T cells, with or without stimulation (Figure 1A). To compare the expression changes before and after EZH2 overexpression, a Wilcoxon test was performed. *P*-values of less than 0.05 were considered statistically significant.

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Results

EZH2, H3K27me3, and miRNA expression in lupus. We first examined the expression of EZH2 in stimulated CD4+ T cells isolated from healthy controls and lupus patients. At the protein level, an increase in EZH2 in lupus T cells was observed compared to control T cells (Figure 1A). In addition, T cell stimulation led to significant increase in EZH2 in lupus T cells compared to controls. Significant increase in *EZH2* mRNA was also observed in lupus patients compared to controls (Figure 1B). Since EZH2 tri-methylates histone H3K27, we measured this histone mark by Western blotting. As shown in Figure 1C, there was a significant increase in H3K27me3 in stimulated lupus naïve CD4+ T cells compared to controls, consistent with the increase in EZH2 in the lupus samples. As both miR-26a and miR-101 control EZH2 expression (15), we then examined the expression of these miRNAs in overnight stimulated control and lupus naïve CD4+ T cells. Both miR-26a and miR-101 were significantly reduced in lupus T cells compared to controls (Figure 1D and 1E). The reduction of these miRNAs in lupus T cells is consistent with and could potentially explain the higher levels of EZH2 observed in lupus compared to healthy controls.

EZH2 expression is controlled by miR-26a and miR-101 in naïve CD4+ T cells. To confirm the role of miR-26a and miR-101 on EZH2 expression in naïve CD4+ T cells, we overexpressed either miR-26a or miR-101 in naïve CD4+ T cells isolated from healthy controls followed by *in vitro* stimulation. Successful overexpression of miR-26a (Figure 2A) or miR-101 (Figure 2B) was first confirmed by TaqMan Advanced miRNA assays. Overexpression of miR-26a or miR-101 indeed decreased EZH2 expression in stimulated CD4+ T cells (Figure 2C). We also overexpressed both miR-26a and miR-101 simultaneously, hypothesizing that the two miRNAs act synergistically to mediate EZH2 expression. Surprisingly, miR-101 appeared to negatively impact miR-26a expression, as simultaneous overexpression of both miRNAs in CD4+ T cells led to significantly lower miR-26a levels compared to overexpression of miR-26a alone (40.1 ± 26.4 fold increase in miR-26a levels in miR-26a-overexpressed T cells vs. 13.9 ± 8.0 fold increase in miR-26a and miR-101-overexpressed T cells, $p<0.04$, $n=6$).

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Overexpression of EZH2 affects genome-wide DNA methylation in naïve CD4+ T cells. To examine the effect of EZH2 on CD4+ T cells, EZH2 was overexpressed in naïve CD4+ T cells isolated from healthy subjects. Successful overexpression of EZH2 was confirmed at both mRNA (Figure 3A) and protein levels (Figure 3B). Overexpression of EZH2 also led to increased H3K27me3 levels (Figure 3B). We then evaluated DNA methylation changes in control or EZH2-overexpressing naïve CD4+ T cells, with or without overnight stimulation. In unstimulated cells, we identified 19 differentially methylated CpG sites in EZH2-overexpressing naïve CD4+ T cells (Supplementary Table 1): eight were hypermethylated and 11 were hypomethylated compared to control-transfected CD4+ T cells, corresponding to 5 and 8 differentially methylated unique genes, respectively. In stimulated cells, a total of 324 differentially methylated CpG sites in EZH2-overexpressing CD4+ T cells were identified (Supplementary Table 1). Among them, 156 were hypomethylated and 168 were hypermethylated compared to control-transfected cells. A total of 242 differentially methylated genes were identified in EZH2-transfected CD4+ T cells following stimulation, with 52% of them being hypomethylated. The top hypomethylated gene in both unstimulated and stimulated EZH2-overexpressing CD4+ T cells was EZH2 (Supplementary Table 1). This reflects overexpression of EZH2 in our experiments, since the two hypomethylated CpG sites in *EZH2* are located in the gene body suggesting that these are from the transfected EZH2 vectors. In addition to *EZH2*, hypomethylation in *IL34* (interleukin-34) was the only gene that was common between the unstimulated and stimulated EZH2-overexpressing CD4+ T cells. We then used DAVID for gene function enrichment analysis to group the differentially methylated genes into Gene Ontologies and biologic pathways. The results showing top pathways and Gene Ontologies enriched among genes significantly hypomethylated with EZH2 overexpression are summarized in Table 1. "Cell adhesion molecules" and "leukocyte migration" appeared to be enriched in both the pathway and Gene Ontology analyses. The genes that overlapped in both included *F11R*, *SELP*, and *SELPLG*. *F11R* encodes junctional adhesion molecule-A (JAM-A), *SELP* codes for p-selectin, and *SELPLG* codes for p-selectin glycoprotein ligand-1 (PSGL1). Since p-selectin is predominantly expressed on endothelial cells to mediate leukocyte adhesion through interaction with

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PSGL1 (16), we focused our subsequent studies on *F11R* and *SELPLG* in naïve CD4+ T cells. Interestingly *SELPLG* was hypermethylated in unstimulated EZH2-overexpressing CD4+ T cells while hypomethylated after stimulation with anti-CD3 and CD28 antibodies. However the expression of PSGL1 remained unaltered with EZH2 overexpression in stimulated CD4+ T cells (data not shown), while JAM-A expression was significantly increased (Figure 3B). In addition, JAM-A was also significantly upregulated in stimulated CD4+ T cells from lupus patients compared to healthy controls (Figure 3C).

Since EZH2 overexpression led to significant changes in the DNA methylome in CD4+ T cells, we examined whether EZH2 affects the expression of enzymes and proteins that are directly involved in DNA methylation. Indeed, in EZH2-overexpressing cells, the expression of both MeCP2 (Figure 3D) and DNMT3A (Figure 3F) was significantly lower compared to control-transfected T cells, while DNMT1 expression was not affected (Figure 3E).

EZH2 mediates CD4+ T cell adhesion to endothelial cells in lupus. Since our pathway and GO analysis pointed to the involvement of EZH2 in leukocyte adhesion and migration (Table 1), we examined whether overexpression of EZH2 affects cell adhesion of stimulated CD4+ T cells to HMVECs. As shown in Figure 4A, overexpression of EZH2 increased T cells adherence to HMVECs. Since JAM-A was hypomethylated and upregulated in EZH2-overexpressing naïve CD4+ T cells (Figure 3B), we examined if increased adhesion in these cells was mediated by JAM-A. When JAM-A was blocked with neutralizing antibodies, the increased adhesion of EZH2-overexpressing CD4+ T cells to HMVECs was normalized (Figure 4B). Since we showed that EZH2 is overexpressed in lupus naïve CD4+ T cells following stimulation (Figure 1), we hypothesized that these cells behave similar to EZH2-overexpressing CD4+ T cells in terms of cell adhesion. Indeed, stimulated lupus CD4+ T cells showed enhanced cell adhesion to HMVECs compared to healthy controls (Figure 4C). Since we also observed increased JAM-A expression in lupus CD4+ T cells (Figure 3C), we blocked JAM-A and showed that cell adhesion of lupus naïve CD4+ T cells was

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significantly reduced (Figure 4D). These results suggest that EZH2 mediates increased T cell adhesion in lupus via JAM-A overexpression. To determine whether inhibition of EZH2 can normalize T cell adhesion in lupus, we used DZNep, an EZH2 inhibitor. DZNep treatment decreased EZH2 and JAM-A expression, and H3K27me3 levels (Figure 4E and 4F). As shown in Figure 4G and 4H, treating stimulated CD4+ T cells from lupus patients with DZNep significantly reduced their ability to adhere to HMVECs. To validate the effect of DZNep on T cell adhesion and determine if this effect is H3K27me3 dependent, we used GSK126, which only inhibits the enzymatic activity of EZH2 but not EZH2 expression. Similar to what we observed in DZNep-treated cells, GSK126 led to significant reduction in lupus T cell adhesion to HMVECs (Figure 4I), suggesting that EZH2-mediated T cell adhesion in lupus is dependent on the catalytic activity of EZH2.

Discussion

In this study, we focused on the expression and function of EZH2 in lupus CD4+ T cells, and identified a novel role for EZH2 in T cell adhesion. We previously demonstrated that this epigenetic modulator might be mediating a pro-inflammatory epigenetic reprogramming in naïve CD4+ T cells in lupus patients upon increase in disease activity (10). Here we demonstrate increased EZH2 expression and H3K27me3 in naïve CD4+ T cells obtained from lupus patients compared to healthy controls following *in vitro* stimulation. In addition, both miR-26a and miR-101, which target EZH2, were significantly downregulated in stimulated lupus naïve CD4+ T cells. Overexpression of EZH2 in naïve CD4+ T cells from healthy individuals resulted in genome-wide DNA methylation changes, and functional enrichment analysis of affected genes led us to examine the role of EZH2 on T cell adhesion. We demonstrated that *in vitro* stimulated CD4+ T cells, either isolated from lupus patients or EZH2-overexpressing cells from healthy subjects, showed increased T cell adhesion to HMVECs compared to their corresponding controls. This was mediated through increased expression of JAM-A in both cases. Blockade of EZH2 by DZNep and GSK126 efficiently blunted lupus T cell

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adhesion to HMVECs, suggesting that blocking EZH2 may be a therapeutic option for lupus.

As the catalytic component of a multi-protein complex polycomb repressive complex 2 that catalyzes H3K27me3, EZH2 is involved in chromatin compaction and gene repression. When phosphorylated, it acts as a transcriptional activator instead and this function is independent of its methyltransferase activity (17). Although EZH2 preferentially targets histones, it has been shown to serves as a recruitment platform for DNMTs to directly control DNA methylation and exert their gene repression activity concurrently (18, 19). In this study we showed that EZH2 overexpression led to genome-wide changes in DNA methylation. In addition to genes involved in cell adhesion and leukocyte migration as highlighted in this study, we also discovered genes that showed differential methylation changes after EZH2 overexpression that are involved in gene transcription (such as *EMX1* and *TAF1*)*,* ubiquitination processes *(BTRC and NEDD4L),* as well as immune response (*CTLA4*, *IL34*, *HLA-E, OAS2,* and *PGLYRP4)* (Table 1 and Supplemental Table 1). These findings suggest that EZH2 is involved in various cellular and physiological processes crucial for T cell survival and function. The exact mechanism of how EZH2 alters the methylome in naïve CD4+ T cells is not clear. However, we show that overexpression of EZH2 leads to repression of both DNMT3A and MeCP2 in naïve CD4+ T cells, which might result in reduced locus-specific DNA methylation or increased chromatin accessibility. In addition, the methylcytosine dioxygenase TET1 was hypermethylated in EZH2-overexpressing CD4+ T cells (Supplemental Table 1), The bivalent repressive/activating effect of EZH2 could explain the close numbers between differentially hyper- and hypomethylated CpG sites (168 vs. 156) after EZH2-overexpression in naïve CD4+ T cells.

Although the function of EZH2 in T cell differentiation remains controversial, it is clear that this pleiotropic enzyme plays a key role in maintaining T cell homeostasis (15, 20-22). Recently, a new function for EZH2 has been revealed. In dendritic cells and neutrophils, EZH2 was shown to control cell adhesion and migration through direct methylation of talin, which directly links integrins to the actin cytoskeleton (23). In our

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current study we also showed that EZH2 mediates cell adhesion, albeit in a different cell type and with a different mechanism. Our data suggest that EZH2 promotes T cell adhesion to endothelial cells, at least in part, via demethylation and upregulation of JAM-A. This is true for naïve CD4+ T cells overexpressing EZH2, and also in lupus naïve CD4+ T cells, which show higher EZH2 expression.

Despite our findings, the exact role of EZH2 in lupus T cells is yet to be fully understood. The role of EZH2 in T cell differentiation and invasiveness is potentially crucial for lupus pathogenesis. EZH2 expression was previously determined in total CD4+ T cells from lupus patients and healthy controls (24). The authors showed that EZH2 mRNA levels were significantly downregulated in total CD4+ T cells from both active and inactive lupus patients. In contrast we showed that EZH2 was elevated in naïve CD4+ T cells from lupus patients compared to healthy controls. The discrepancy might arise from the different T cell population that was studied.

The process of T cell entry into inflamed tissues involves multiple steps, including morphological changes of T cells, adherence of the freely circulating cells to the endothelium, and subsequent migration into the tissue. In lupus, infiltration of T cells into inflamed tissue has been well documented (25, 26). Increased endothelial cell activation, represented by elevated expression of E-selectin, VCAM-1, and ICAM-1, to increase their adhesiveness to leukocytes has been reported in lupus skin (27). In lupus lymphocytes increased LFA-1 and VLA-4 was also reported (28). Li et al. reported that lupus T cells display a higher ability to adhere to hyaluronic acid-coated plates, which is mediated by increased phosphorylated ezrin, radixin, and moesin (ERM) and increased expression of CD44 (29). Our findings identify JAM-A as a novel molecule involved in increased cell adhesion and possibly tissue invasiveness of lupus CD4+ T cells.

JAM-A is a member of the immunoglobulin supergene family that has been identified at tight junctions between endothelial cells and epithelial cells. It is also expressed on some circulating cells including lymphocytes (30). JAM-A is engaged in a number of binding interactions: hemophilic interactions linking adjacent endothelial and epithelial

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cells to stabilize intercellular junctions; or heterophilic interactions with leukocyte integrins or JAMs to facilitate leukocyte influx during inflammation (31, 32). The importance of JAM-A in leukocyte-endothelial cell interactions is highlighted by the work using JAM-A-deficient mice and neutralizing antibodies to JAM-A (33-35). To our knowledge, we are the first to report JAM-A to be elevated in lupus CD4+ T cells and to show that it mediates increased T cell adhesion to endothelial cells. In other autoimmune diseases, dysregulated expression of JAM-A has been reported (36). Decreased expression of JAM-A was observed in scleroderma skin endothelial cells while increased expression of JAM-A was reported in scleroderma fibroblasts, which mediated myeloid cell retention on scleroderma skin (36). In peripheral blood mononuclear cells isolated from rheumatoid arthritis patients, *F11R* was hypomethylated, and an upregulation of *F11R* was observed (37, 38), similar to what we observed in our study.

In summary, we report a novel role for EZH2 in T cell adhesion, and a potentially pathogenic role for EZH2 overexpression in lupus. Upregulation of EZH2 in lupus CD4+ T cells, possibly mediated by downregulation of miR-26a and miR-101, results in increase in H3K27me3 levels. EZH2 overexpression in T cells alters the DNA methylome and highlights the involvement of EZH2 in leukocyte adhesion and migration, via upregulation of JAM-A. We show that increased lupus CD4+ T cell adhesion is mediated by JAM-A overexpression, and that blockade of JAM-A or EZH2 reduces the ability of CD4+ T cells to adhere to endothelial cells. These results suggest inhibiting JAM-A or EZH2 as potential novel therapeutic approaches in lupus.

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Figure legends

Figure 1. The expression of EZH2, miRNAs, and H3K27me3 in CD4+ T cells isolated from healthy controls and lupus patients. (A) At the protein level, EZH2 was significantly elevated in naïve CD4+ T cells from lupus patients (86.2 ± 57.1, n=3) compared to control cells (14.0 ± 11.5, n=4) after overnight stimulation with anti-CD3 and anti-CD28 antibodies (p=0.02 from two-way ANOVA, post-hoc analysis p=0.02). Stimulation led to significant increase in EZH2 in lupus T cells (unstimulated vs. stimulated: 15.2 ± 7.6 vs. 86.2 ± 57.1, n=3, post-hoc analysis p=0.04). (B) The expression of EZH2 mRNA was

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significantly elevated in naïve CD4+ T cells from lupus patients compared to healthy controls after overnight stimulation with anti-CD3 and anti-CD28 antibodies (n=13-16, control vs. SLE 0.011 ± 0.004 vs. 0.018 ± 0.009; p=0.02). (C) H3K27me3 was significantly elevated in lupus naïve CD4+ T cells (n=9, 0.336 ± 0.216) stimulated overnight compared to cells from healthy controls (n=7, 0.111 ± 0.054, p=0.04). (D-E) Both miR-26a and miR-101 were reduced in stimulated lupus CD4+ T cells (miR-26a: control [n=14] vs. SLE [n=13]: 0.047 ± 0.029 vs. 0.021 ± 0.018, p=0.01; miR-101: control [n=14] vs. SLE [n=12]: 0.016 ± 0.011 vs. 0.008 ± 0.006, p=0.03). Results are expressed as mean +/- SD and p<0.05 was considered significant.

Figure 2. Overexpression of miR-26a or miR-101 reduced EZH2 levels in stimulated CD4+ T cells. (A-B) Confirmation of overexpression of miR-26a (n=3, 0.021 ± 0.002 vs.1.049 ± 0.560 vs. 0.025 ± 0.011 for control vs. miR-26a vs. miR-101, p=0.01 by one-way ANOVA, post-hoc analysis control vs. miR-26a p=0.02) and miR-101 (n=3, 0.017 ± 0.007 vs. 0.023 ± 0.010 vs. 1.173 ± 0.613 for control vs. miR-26a vs. miR-101, p=0.01 by one-way ANOVA, post-hoc analysis control vs. miR-101 p=0.02) in CD4+ T cells. (C) Overexpression of either miR-26A (n=3) or miR-101 (n=3) reduced EZH2 levels significantly in CD4+ T cells (0.76 ± 0.08 and 0.79 ± 0.05 for miR-26a and miR-101 normalized to control, p=0.003 by one-way ANOVA, post-hoc analysis p=0.003 for control vs. miR-26a; p=0.006 for control vs. miR-101). Results are expressed as mean +/- SD and p<0.05 was considered significant.

Figure 3. Overexpression of EZH2 in stimulated CD4+ T cells affects JAM-A expression and enzymes and proteins involved in DNA methylation. (A) Overexpression of EZH2 in control CD4+ T cells was confirmed by qPCR (0.016 ± 0.008 vs. 0.525 ± 1.225 for control vs. EZH2 vector, n=14, p=0.0001). (B) EZH2 overexpression led to increase in JAM-A and H3K27me3 expression in CD4+ T cells. Representative figure of n=3. (C) JAM-A was overexpressed in naïve CD4+ T cells from lupus patients compared to control after overnight stimulation (densitometry analysis: Controls 0.0026 ± 0.0002 vs. lupus 0.0038 ± 0.0009; p=0.047, n=4). (D-F) EZH2 overexpression reduced the expression of MeCP2 (n=14, 0.003 ± 0.002 vs. 0.002 ± 0.001 control vs. EZH2 vector,

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p=0.04) and DNMT3A (n=14, 0.003 ± 0.001 vs. 0.002 ± 0.001 control vs. EZH2 vector, p=0.02), but had no effect on DNMT1 (n=14, 0.006 ± 0.002 vs. 0.006 ± 0.002 control vs. EZH2 vector, p=0.42). Results are expressed as mean +/- SD and p<0.05 was considered significant.

Figure 4. Effect of EZH2 on lymphocyte adhesion. (A) EZH2-overexpressing stimulated CD4+ T cells showed increased adhesion to HMVECs compared to cells transfected with an empty vector (n=8, control vs. EZH2 vector 1.0 ± 0.0 vs. 1.94 ± 0.74, p=0.02). (B) The increased adhesion of EZH2-overexpressing CD4+ T cells was blocked by neutralizing antibodies to JAM-A (One-way ANOVA p=0.002; n=6, control vector: IgG vs. JAM-A 1.00 ± 0.09 vs. 0.92 ± 0.15, post-hoc p=0.99; EZH2 vector: IgG vs. JAM-A 1.77 ± 0.57 vs. 1.04 ± 0.38, post-hoc p=0.01. Control vector IgG group vs. EZH2 vector IgG group post-hoc p=0.008). (C) Naïve CD4+ T cells from lupus patients stimulated overnight adhere to HMVECs significantly more than cells from healthy controls (n=6, control vs. SLE 1.0 ± 0.0 vs. 2.43 ± 2.31, p=0.002). (D) Blocking JAM-A reduced the ability of lupus CD4+ T cells to adhere to HMVECs (One-way ANOVA p=0.007; n=6, control: IgG vs. JAM-A 1.00 ± 0.09 vs. 1.05 ± 0.38, p=1.00; SLE: IgG vs. JAM-A 2.19 ± 1.08 vs. 1.12 ± 0.31, p=0.03. Control IgG group vs. SLE IgG group p=0.01). (E) Treating stimulated lupus CD4+ T cells with EZH2 inhibitor DZNep reduced EZH2 mRNA levels significantly (n=5, PBS vs. DZNep 0.013 ± 0.005 vs. 0.005 ± 0.002, p=0.02]. (F) At the protein levels, DZNep decreased EZH2, JAM-A, H3K27me3 levels in stimulated lupus CD4+ T cells. (G) Pre-treating stimulated CD4+ T cells from lupus patients with DZNep for 3 days significantly reduced their adhesion to HMVECs compared to cells treated with PBS (n=6, PBS vs. DZNep: 1.0 ± 0.0 vs. 0.42 ± 0.21, p=0.002). (H) Representative pictures of the adhesion assay: stimulated CD4+ T cells (in green) isolated from a lupus patients showed increased ability to adhere to HMVECs (in red) compared to a healthy control, and DZNep significantly reduced adhesion of CD4+ T cells from the same lupus patient. (I) Pre-treating stimulated CD4+ T cells isolated from lupus patients with GSK126 for 3 days led to significant reduction in cell adhesion compared to cells treated with DMSO (n=6, DMSO vs. GSK126: 1.0 ± 0.0 vs. 0.47 ± 0.32, p=0.048). Results are expressed as mean +/- SD and p<0.05 was considered significant.

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Table 1: Functional annotation pathway and Gene Ontology analysis of genes hypomethylated with EZH2 overexpression in *in vitro* stimulated CD4+ T cells.

Pathway analysis					
KEGG_PATHWAY	Ubiquitin mediated proteolysis	*BTRC, TCEB2, NEDD4L, UBE2D1, UBE2R2*	0.01	5.17	15.58
KEGG_PATHWAY	Cell adhesion molecules	*F11R, SELP, NTNG2,*	0.02	4.95	17.79

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		HLA-E, SELPLG			
KEGG_PATHWAY	Staphylococcus aureus infection	*C3AR1, SELP, SELPLG*	0.05	8.27	44.16
Gene Ontology analysis					
GOTERM_BP_DIRECT	GO:0007596~blood coagulation	*ATP2B1, F11R, SELP, STX4, P2RX1, KIFAP3, C6ORF25, PRKCE, SIRPA, SELPLG*	0.00	3.24	5.25
GOTERM_BP_DIRECT	GO:0018171~peptidyl-cysteine oxidation	*TMX3, PRDX3*	0.01	159.34	17.28
GOTERM_BP_DIRECT	GO:0006355~regulation of transcription, DNA-templated	*EMX1, EID2B, EZH2, MITF, ZBTB41, ZNF250, IRF2BP2, RORA, WWTR1, ZNF629, ZNF747, HNRNPUL1, NR1D2, SP3, CDK11B, SPATA24*	0.01	1.98	18.38
GOTERM_BP_DIRECT	GO:0010467~gene expression	*TAF1, EZH2, LARS2, RORA, WWTR1, WARS, HNRNPUL1, NR1D2, TCEB2, MARS2, HIST1H4C, NEDD4L, UBE2D1*	0.02	2.16	21.30
GOTERM_BP_DIRECT	GO:0006469~negative regulation of protein kinase activity	*SOCS2, PRKRIP1, TRIM27, WWTR1*	0.02	7.41	22.29
GOTERM_BP_DIRECT	GO:0006367~transcription initiation from RNA polymerase II promoter	*TAF1, NR1D2, NEDD4L, RORA, UBE2D1, WWTR1*	0.02	3.81	26.68
GOTERM_BP_DIRECT	GO:0045599~negative regulation of fat cell differentiation	*RORA, WWTR1, TCF7L2*	0.03	11.95	32.60
GOTERM_BP_DIRECT	GO:0006418~tRNA	*WARS, MARS2,*	0.03	11.38	35.11

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	aminoacylation for protein translation	*LARS2*			
GOTERM_BP_DIRECT	GO:0070936~protein K48-linked ubiquitination	*NEDD4L, UBE2D1, UBE2R2*	0.03	10.17	41.38
GOTERM_BP_DIRECT	GO:0000122~negative regulation of transcription from RNA polymerase II promoter	*ZMYND11, SP3, MITF, EZH2, TRIM27, NEDD4L, FOXO3, UBE2D1, WWTR1, TCF7L2*	0.04	2.21	42.16
GOTERM_BP_DIRECT	GO:0050900~leukocyte migration	*F11R, SELP, SIRPA, SELPLG*	0.04	5.49	42.45
GOTERM_BP_DIRECT	GO:2000650~negative regulation of sodium ion transmembrane transporter activity	*NEDD4L, PRKCE*	0.04	53.11	43.40
GOTERM_BP_DIRECT	GO:0050885~neuromuscular process controlling balance	*BCR, TNR, CLIC5*	0.04	9.76	43.85
GOTERM_BP_DIRECT	GO:0042752~regulation of circadian rhythm	*NR1D2, BTRC, EZH2*	0.04	9.76	43.85
GOTERM_BP_DIRECT	GO:0071456~cellular response to hypoxia	*TCEB2, RORA, PRKCE, UBE2D1*	0.04	5.36	44.55
GOTERM_BP_DIRECT	GO:0030168~platelet activation	*SELP, STX4, P2RX1, C6ORF25, PRKCE*	0.05	3.67	51.46
GOTERM_BP_DIRECT	GO:0042753~positive regulation of circadian rhythm	*BTRC, RORA*	0.05	39.83	53.19

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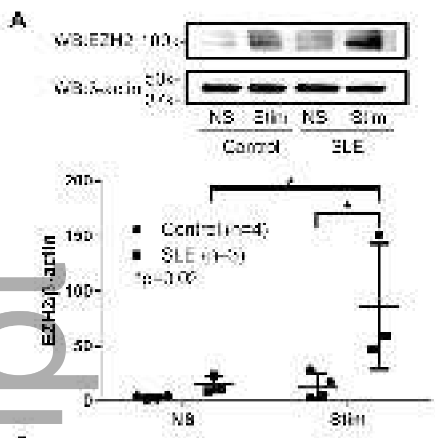

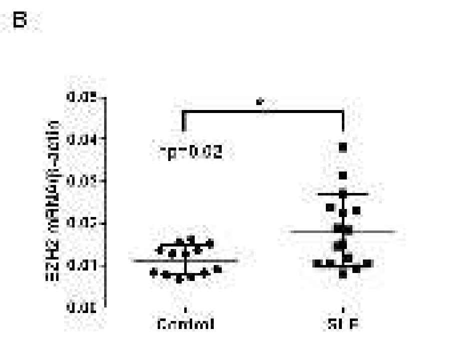

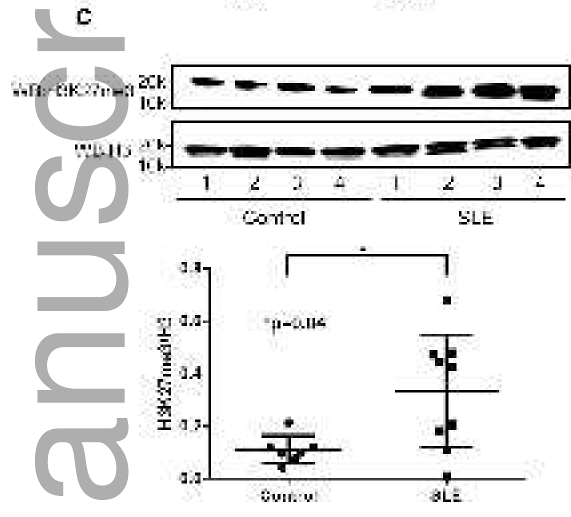

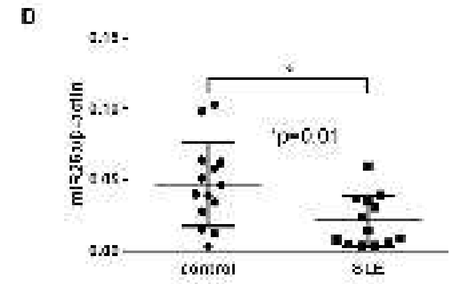

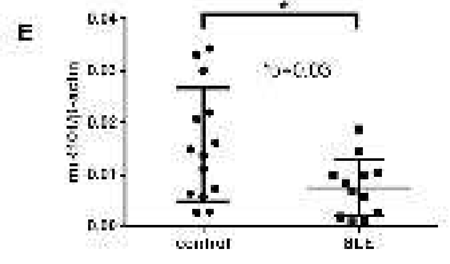

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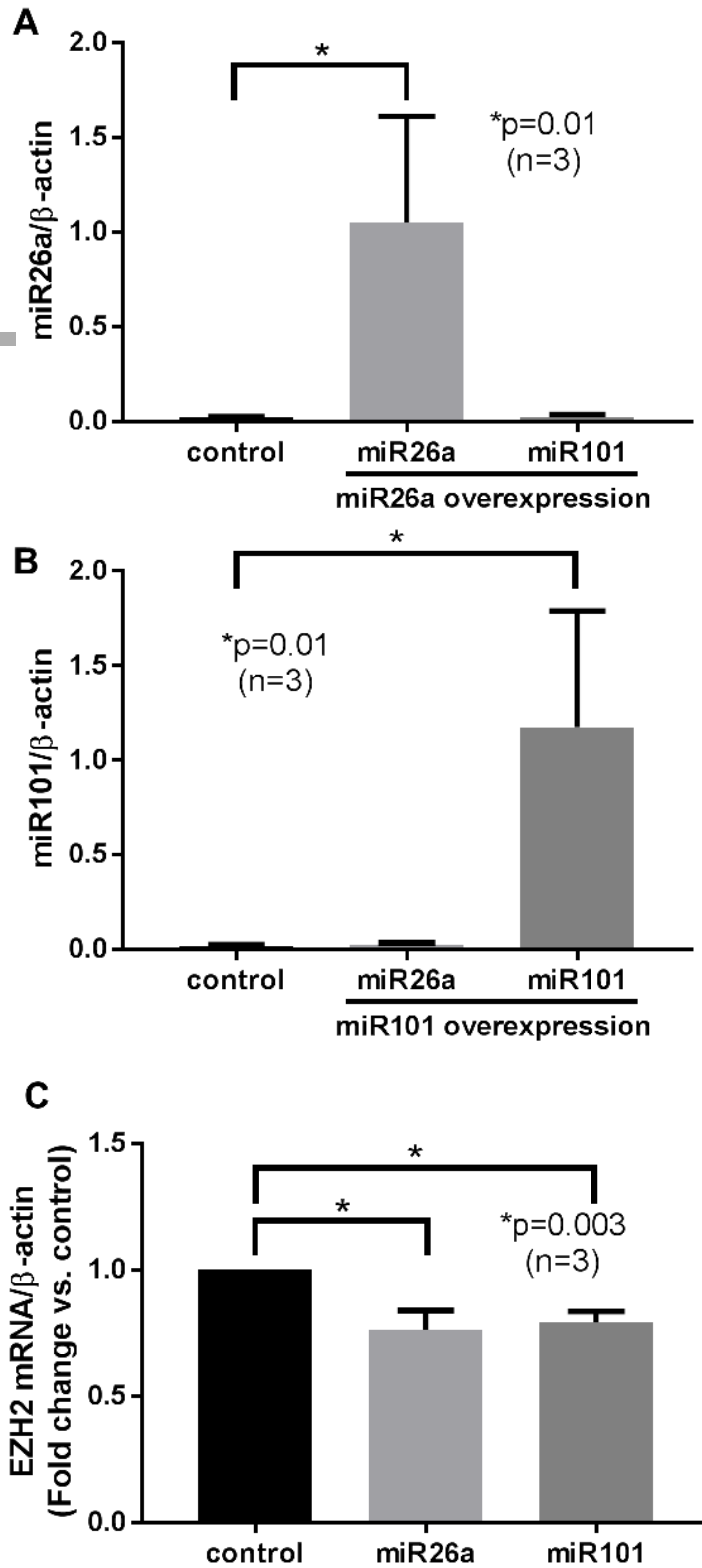

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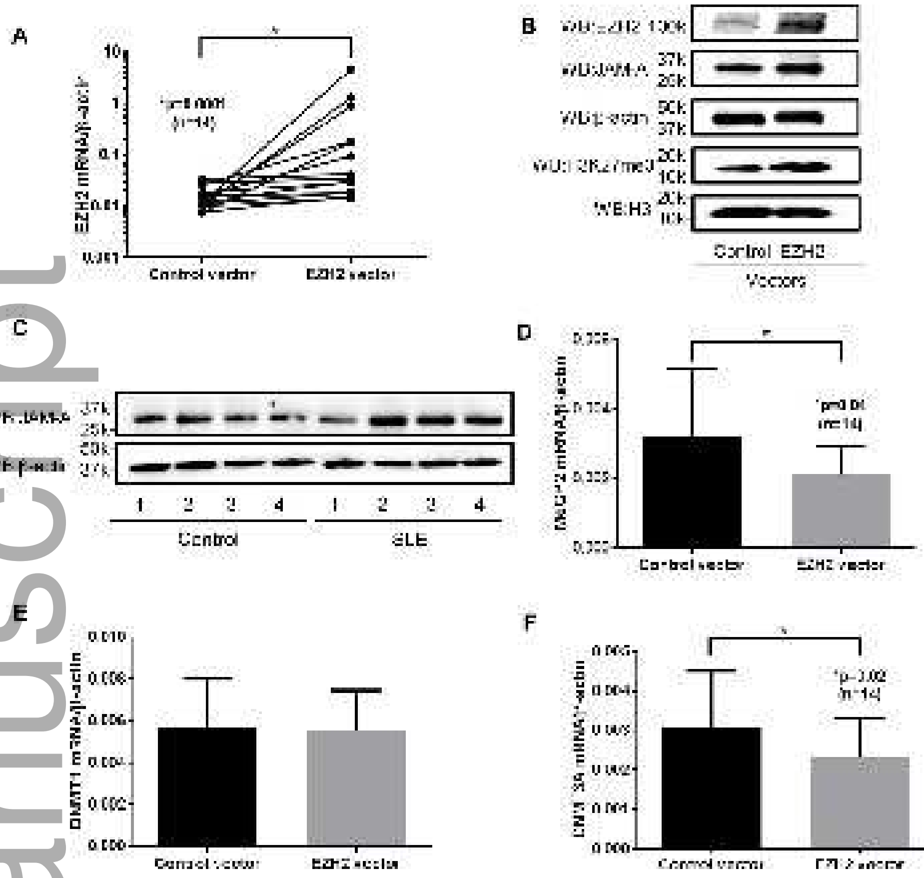

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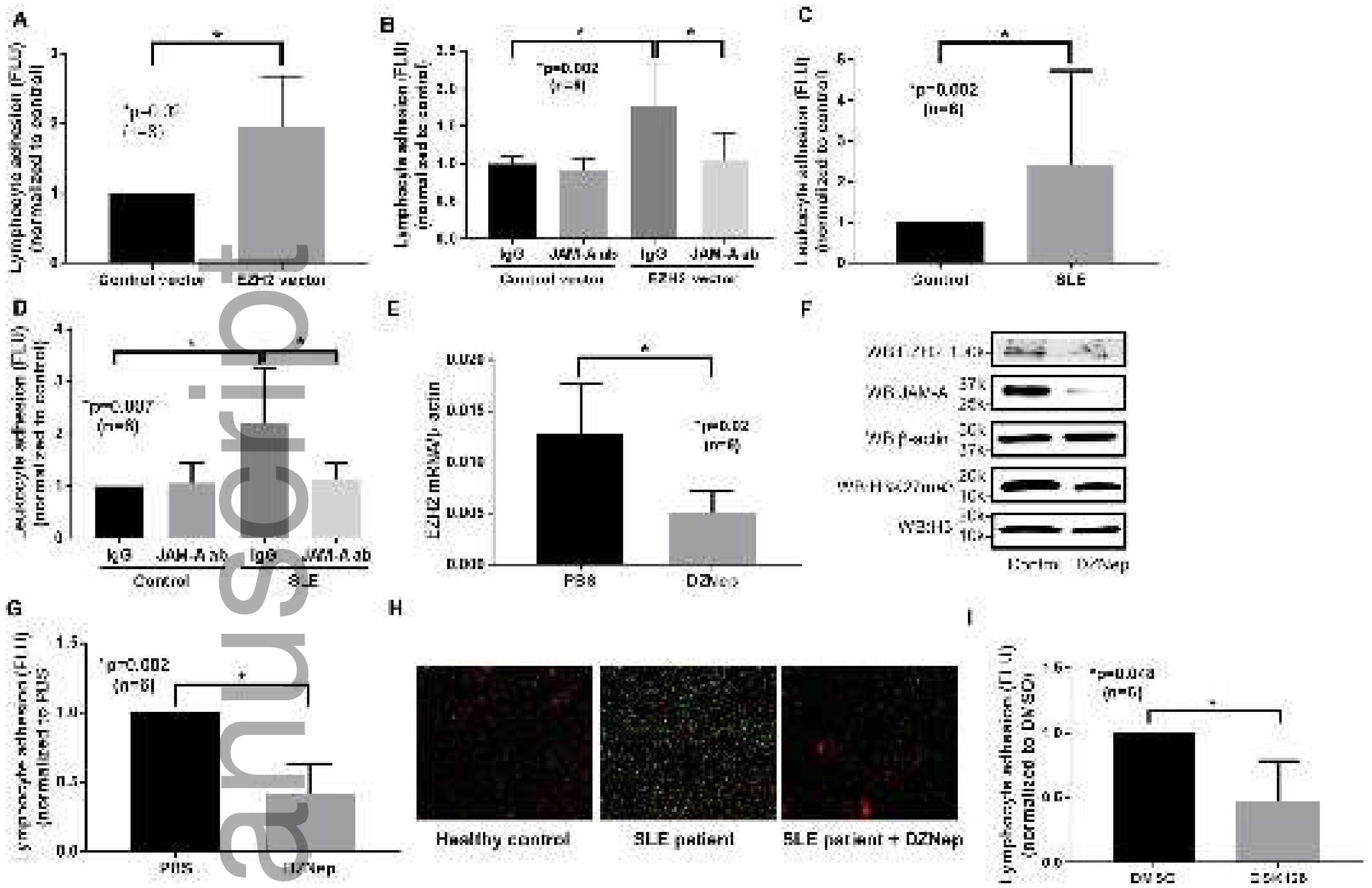

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