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Overall Status of Epstein-Barr Virus Infection, IFN-a, and TLR-7/9 in Patients with Systemic Lupus Erythematous

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ABSTRACT

Background: Systemic lupus erythematous (SLE) is a multisystem autoimmune disorder. While studying the pathogenesis of SLE is prevalent, both infectious and non-infectious elements are regarded to exert an important impact on the disease's development.

Objective: To explore the overall status of EBV, TLR7, TLR9, and IFN-α gene expression in 32 patients suffering from SLE and 32 healthy controls.

Methods: Plasma and PBMCs were separated from fresh whole blood. To measure EBV DNA load and mRNA levels of IFN-a, TLR-7 and9 in PBMCs, molecular techniques were employed. The production of IFN-α, ds-DNA IgG antibody, and EBNA-1 IgG levels were also measured in plasma by ELISA.

Results: SLE patients showed significantly higher EBV load (P=0.001) and transcriptional levels of TLR7 (P=0.0001), IFN-α (P=0.0001), and TLR9 (P=0.0001) than controls. Moreover, the plasma levels of IFN-α (P=0.0002) and EBNA-1specific IgG antibodies (P=0.01) were significantly higher in SLE patients.

Conclusion: The results stressed on the potential role of EBV infection and TLRs in SLE patients although more research is needed to determine the global impact that EBV infection can have on immune signature in patients with SLE.

Keywords: Epstein-Barr Virus, IFN-α, Systemic Lupus Erythematous, Toll-like Receptors

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INTRODUCTION

Systemic lupus erythematosus (SLE) is a type of heterogeneous disease in which multiorgan are involved and occurs in the second or third decade of life with the female/male ratio of 9:1 (1). SLE disease clinically represents different forms (2) and is mainly marked by the production of autoantibodies, lack of tolerance to self-antigens and dysregulated immune responses such as aberrant regulation of cytokines (3), and long term activation of type I interferons (IFNs) (4), and dysregulation of B cells (5). Given the fact that the majority of SLE patients produce IFN-α constantly, the mechanism behind excessive IFN-α production in these patients has not been defined yet. IFN-α levels were shown to

be linked with the production of anti-double-stranded DNA antibodies (6), disease activity, and severity (7). Moreover, IFN-α enhances antiviral responses and activates the immune system more (8).

While the pathogenesis of SLE is so popular, environmental and genetic factors like the Epstein-Barr virus (EBV) infection are considered to contribute to disease risk (9). Increased frequency of EBV-specific antibodies (10, 11) and elevated viral load (12) in SLE patients highlight its potential role in the disease development. Moreover, higher pervasiveness of EBV infection among young patients may suggest a probable etiology for SLE (13). Systemic lupus erythematous in adults is thought to be linked to have been exposed to EBV previously (14).

Toll-like receptors (*TLRs*) are a class of proteins with a major role in the *innate immune system*. They *trigger* a cascade of signaling pathways, for example, *TLR* signals induce *inflammatory cytokines* and type I *interferons (IFN* α /β) (16). It has been shown that viral infections including EBV can induce IFN-α production (17, 18) through TLRs pathways (19, 20). Moreover, the expression levels of *TLR7*, and *TLR9* remarkably rose in lupus patients (21-25), which further indicates the highlighted contribution of TLRs in SLE. Furthermore, TLRs are suggested to have a key role in the generation of antibodies to RNA-binding proteins (26).

As recently found, the innate immune system, and through engaging TLRs and producing interferon, greatly controls viral infections. Excessive production of IFN-α observed in SLE patients is thought to be because of the chronic viral infection that has been abnormally controlled (17). Indeed, Bents et al., found that latent membrane protein-1 (LMP1) can regulate the function of IFN regulatory factors (27). Moreover, LMP1 was shown to be positively correlated with *IFN*-stimulated genes in these patients (28).

Considering the highlighted importance of IFN-α in SLE pathogenesis and the potential role of EBV infection in both SLE disease and IFN-α production, the present paper aims at studying the overall status of EBV, *TLR7*, *TLR9*, and *IFN-α* gene expression in patients suffering from SLE and in healthy controls.

PATIENTS AND METHODS

Study Population

32 SLE patients and 32 age and sex-matched healthy were included here. Patients were identified based on the criteria presented by ACR (American College of Rheumatology) (29), and the clinical picture was assessed by clinicians at the Rheumatology Research Center. Patients with other forms of lupus except for the systemic form and those who suffer from another kind of autoimmune diseases related to EBV (such as Rheumatoid arthritis, Sjögren syndrome, Autoimmune thyroiditis, Autoimmune liver disease) (30) were excluded from this study. All research participants must give their permission to be part of a study. We collected whole blood (*10 ml*) from all research participants. Ethics approval for the study was obtained (approval code: IR. TUMS. SPH. REC. 1395. 949) from Tehran University Medical school.

PBMCs Separation

PBMCs are separated from 10 ml whole blood by a density gradient centrifugation method using *Fico'll*-Paque (GE Healthcare, Amersham, UK). Isolated PBMC were washed twice with RPMI 1640 (Gibco Life Technologies, USA) and re-suspended in the same medium supplemented with 10% fetal bovine serum (Gibco Life Technologies, USA).

RNA Extraction and cDNA Synthesis

RNA from PBMCs was manually extracted by Trizol as the manufacturer (Thermo Fisher Scientific, Wilmington, DE, USA) instructed. After the dissolution of the final RNA pellet in 30µl of DEPC water, the concentration and purity of total RNA were determined by using gel electrophoresis and NanoDrop ND-1000

spectrophotometer (Thermo Fisher Scientific), respectively. 500ng of total RNA was utilized in synthesizing cDNA for a 20µl total reaction as instructed by the manufacturer (GeNet Bio).

Measurement of mRNAs Level in PBMCs

A 10-fold dilution series was employed to evaluate the efficiency of real-time PCR through analyzing amplification and melting curves of *GAPDH, IFN-α*, and *TLR7,9*. Based on our initial setup experiments, a 1:10 dilution sample was included in SYBR Green qPCR assays. The primers utilized for the Real-time PCR assay were: A 10-fold dilution to evaluate the efficiency of real-time PCR through analyzing amplification and melting curves of *GAPDH, IFN-α,* and *TLR9*. Based on our initial setup experiments, a 1:10 dilution sample was included in SYBR Green qPCR assays. The primers utilized for the Real-time PCR assay were *GAPDH* (Forward: TCCAAAATCAAGTGGGGCGA Reverse: TGATGACCCTTTTGGCTCCC), *IFN-α* (Forward: AACTCCCCTGATGAATGCGG Reverse: CTGCTCTGACAACCTCCCAG), *TLR9* (Forward: GGAGATGGTGCCTACAAGGG Reverse: CTGGATAGCACCAGTAGCGG), and *TLR7* (Forward: CCTTGTGCGCCGTGTAAAAA Reverse: GGGCACATGCTGAAGAGAGT).

Relative quantitative real-time PCR was employed to determine the mRNA levels of *IFN-α, TLR7,* and *TLR-9* using 2X SYBR® Green ER™ qPCR SuperMix Universal (Roche) on the Rotor-Gene® 6000 (Corbett Research, Sydney, Australia). Amplifications were performed in volumes of 25 µl containing 2.5 µl of target cDNA (10 fold diluted), 12.5 µl of SYBR Green, and 0.5 µmolar of each primer. The accuracy and linearity of real-time PCR were assessed using Glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) mRNA levels in all stimulated groups. Samples were incubated at 95°C for 10 min, then 45 cycles of 95°C for 15 sec, 57°C for 30 sec, followed by 72°C for 30 sec for all primers.

DNA Extraction and EBV Load Quantitation

Given the fact that B cells act as a main source of latency in EBV infection, any changes in the state and the function of B cells could potentially impact EBV infection and the overall status of EBV infection in patients suffering from SLE. As such, EBV load was quantified in PBMC using real-time PCR. A high Pure Viral Nucleic acid kit (Roche, Germany) was employed to extract DNA from PBMCs (5×10^6). Absorbance at 260 nm was measured to determine the concentration of the extracted DNA. The purified *DNA* was eluted in 50µl of elution buffer and stored at -20°C for further use. *EBV* viral load was quantified using RealStar® EBV PCR Kit 1.0 (Altona, Germany). Briefly, 10µl of extracted DNA and 20µl master mix was applied in a total reaction of 30µl. The thermocycling profile for real-time PCR was 10 min at 95 and 15 sec at 95, followed by 45 cycles, with dye acquisition during the 1 min at 58. Four standards of known concentrations were run to generate a standard curve for quantitative analysis. Moreover, internal control was utilized for each sample to identify possible PCR inhibitors and to confirm the reliability of the reagents of the kit. EBV DNA detection limit was defined as values of 10 copy numbers. The virus copy number was finally calculated based on copy/µg.

IFN-α and Anti-dsDNA Determination

IFN-α production was quantified in plasma via Human IFN-α ELISA development kit (Mabtech-Sweden) based on the manufacturer's protocol. A cutoff value was higher than in 1 pg/mL according to the manufacturer's protocol. The plasma levels of Anti-dsDNA were measured employing the ELISA development kit (Euroimmun kit-Germany) and seropositivity was defined as values of ≥100 IU/ml based on the manufacturer's protocol.

Statistical Analysis

Transcriptional levels of *GAPDH, IFN-α, TLR7*, and *TLR9* were analyzed applying

fold change as described previously (31-33). Statistical analysis was conducted through utilizing the Graph Pad Prism software, version 8 (*Graph pad Software Inc* in *La Jolla, CA*). The Mann–Whitney and Student's t- test was utilized for the comparison of data sets which were distributed non-normally and normally, respectively. P-values≤0.05 were set as significant.

RESULTS

Demographic Characteristics

The demographic and clinical characteristics of 32 SLE patients suffering from different forms of the disease are summarized in Table 1. Thirty-two patients (29 females, 3 males; 32 years on average) and 32 healthy control subjects (29 females, 3 males; 32 years on average) were selected. The sex ratio of study subjects was 9:1 and similar age distribution was detected among study subjects. All the patients received treatment, except for four patients considered as 'd drug-naïve'. Treatment was given to twenty-eight patients, either alone or in combination.

Anti-dsDNA Status

As expected, 100% of the SLE patients had

Table 1. Demographic and clinical characteristics of SLE patients

SLE	Age	Disease form	Sex	Prednisolone	Hydroxychloroquine	Azathioprine	SLEDAI.2K Score
1	47	Drug naïve	F	No	No	No	-
2	38	Drug naïve	F	No	No	No	-
3	32	Drug naïve	F	No	No	No	-
4	26	Drug naïve	F	No	No	No	-
5	22	Inactive	F	7.5mg	200mg	No	2
6	26	Inactive	M	5mg	No	50mg	2
7	43	Inactive	F	5mg	200mg	No	2
8	28	Inactive	F	5mg	200mg	No	3
9	40	Inactive	F	2.5mg	200mg	No	3
10	26	Inactive	F	5mg	200mg	50mg	3
11	22	Inactive	F	5mg	200mg	No	3
12	36	Inactive	F	5mg	200mg	50mg	3
13	38	Inactive	F	5mg	200mg	No	3
14	27	Inactive	F	5mg	200mg	No	4
15	28	Inactive	F	2.5mg	100mg	No	4
16	38	Inactive	M	5mg	200mg	No	4
17	35	Inactive	F	5mg	No	50mg	4
18	43	Inactive	F	5mg	200mg	50mg	4
19	25	Inactive	F	5mg	200mg	No	4
20	34	Inactive	F	5mg	No	No	5
21	15	Inactive	F	5mg	200mg	50mg	5
22	27	Inactive	F	5mg	200mg	50mg	5
23	35	Inactive	F	5mg	200mg	No	5
24	43	Inactive	F	7.5mg	No	No	5
25	38	Active	F	5mg	200mg	No	16
26	28	Active	F	5mg	No	50mg	16
27	36	Active	F	5mg	200mg	50mg	18
28	36	Active	F	10mg	200mg	50mg	20
29	31	Active	F	5mg	200mg	No	22
30	32	Active	F	10mg	200mg	50mg	24
31	40	Active	F	5mg	No	No	25
32	18	Active	M	5mg	200mg	50mg	26

F: Female, M: Male, SLEDAI.2K**:** The Systemic Lupus Erythematosus Disease Activity Index 2000

anti-ds-DNA IgG antibodies. As indicated in Figure 1A, the highest concentration was seen in patients suffering from inactive SLE (median, range: 60149, 9461-158416), although the difference was not statistically significant in comparison with the active SLE patients (81686, 1660-151034) and drug-naïve patients (55192, 28180-89334). Moreover, no remarkable difference was observed when the data collected from patients with SLE were analyzed by demographic data and the type of treatment they received.

EBV Status

The level of the EBNA-1 specific IgG antibody was determined in plasma. The EBNA-1 specific IgG titer was considerably higher in SLE patients than in the controls (median, range: 9310, 1-48138 vs 4098, 43-12443) (P=0.01) (Figure 2A). Data from SLE were further analyzed based on disease forms. As indicated in Figure 2B, the EBNA-1specific IgG titer was remarkably higher in patients with inactive SLE than in those suffering from active forms (P=0.04). However, such a difference was not observed

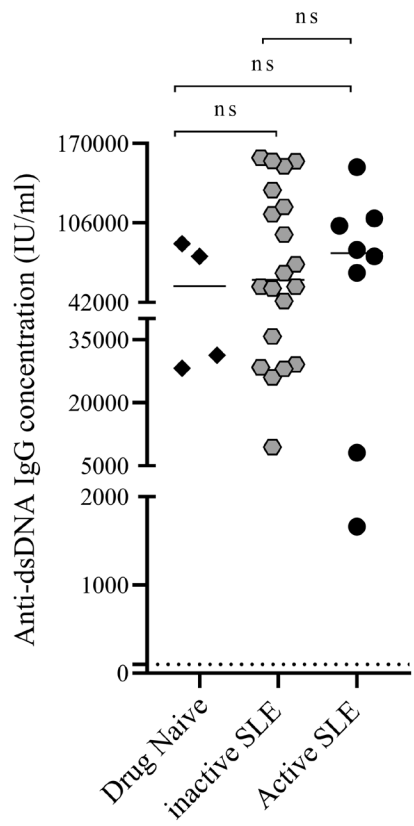

Figure 1. Anti-dsDNA IgG. Dot plots showing plasma level of Anti-dsDNA IgG in SLE patients and healthy controls. Solid lines indicate median. ns indicate non-significant. P-values were identified utilizing the Mann-Whitney test.

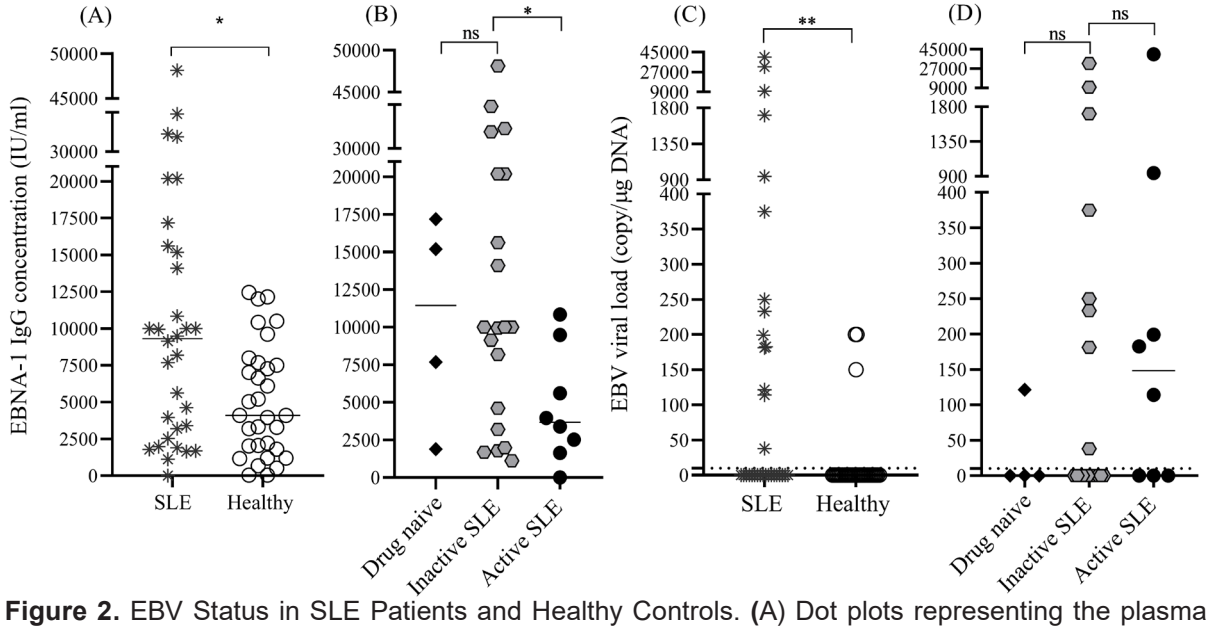

Figure 2. EBV Status in SLE Patients and Healthy Controls. **(**A) Dot plots representing the plasma concentration of the anti-EBNA-1 IgG antibody in SLE patients compared with those of the healthy controls. Dash lines represent median values and cut-off >10 IU/ml. (B) The EBNA-1 specific IgG antibody in SLE groups. (C) The EBV viral load in PBMCs is plotted for patients with SLE and for healthy controls. (D) The EBV viral load from SLE patients was subdivided based on active and inactive forms. Dash lines indicate detection limit. Solid lines indicate median. 1star, 2 stars, and ns indicate P<0.05, P<0.01 and non-significant, respectively. P-values were specified employing the Mann-Whitney test.

when compared with drug-naïve patients (P>0.05). Furthermore, no discernible change was discovered when the data from SLE patients were analyzed following the demographic data and the type of treatment they received.

Considering the potential role of EBV infection in SLE disease, the EBV load was quantified in PBMCs (copy/µg) using real-time PCR. The EBV load was remarkably higher in SLE patients than in the controls (median, range: 0, 0-40580 vs.0, 0-200) copies (P=0.002) (Figure 2C). SLE patients (n=14, 43%) frequently exceed the assay detection limit compared with healthy controls (n=3; 9.37%) (P<0.05). Although the median copy number of the EBV in patients with active form was greater than that of patients with inactive SLE, the difference was not statistically significant (Figure 2D). Moreover, there was no notable difference when the data from SLE patients were analyzed according to demographic data and the type of treatment they received.

IFN-α Status

Looking at the plasma levels of the IFN-α, patients with SLE revealed significantly higher plasma concentration of the IFN-α (median, range: 16.92, 0.42- 392) than the healthy controls (1.59, 0.31- 29.48) (P=0.0002) (Figure 3A). Although the plasma concentrations of the IFN-α in both inactive (15.92, 0.54-392) and drug-naïve (19.15, 18.30-82.48) patients were higher than in active patients (6.18, 0.42-71.56), the groups were not significantly different. (Figure 3B). Moreover, no substantial difference was observed when data from SLE patients were analyzed relying on demographic data and the type of the received treatment.

The IFN-α gene expression on PBMCs was also remarkably higher in SLE patients (median, range: 1.82, 0.291-9.51) than in the controls (0.51, 0.015-3.19) (P<0.0001) (Figure 3C). Looking at the IFN-α gene expression among SLE patients we found that patients with inactive SLE (2.087, 0.353-9.50) induced significantly higher IFN-α gene expression than in drug-naïve patients (0.99, 0.29-1.23) (P=0.02). However, such a difference did not reach a statistically significant level when compared with active patients (1.41, 0.61-3.19)

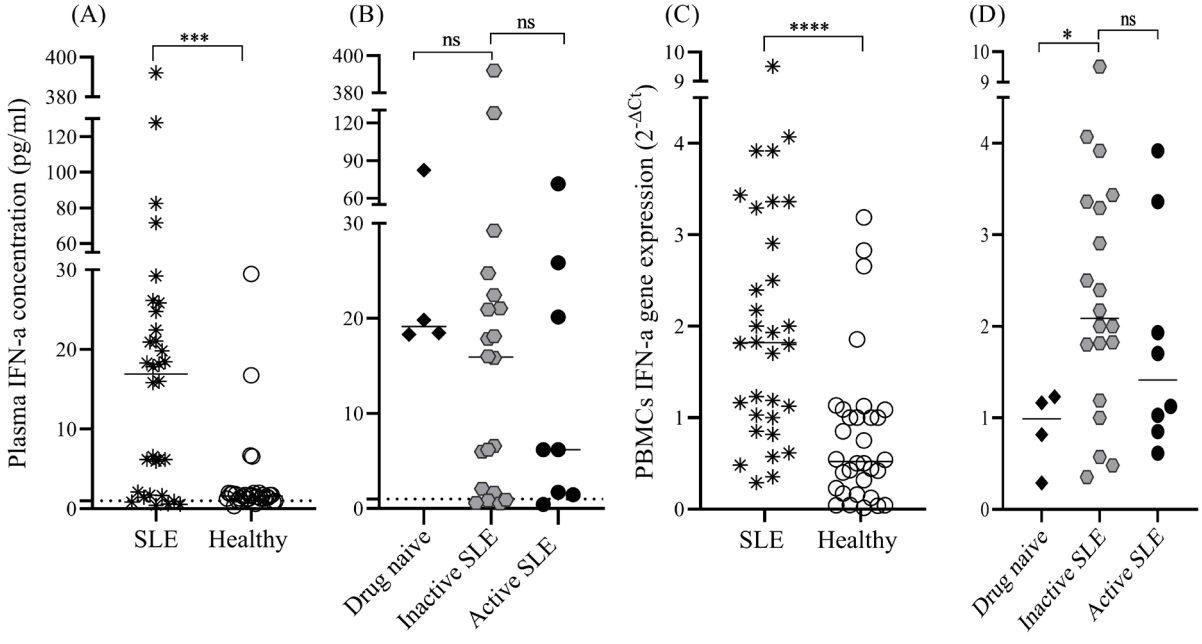

Figure 3. IFN- Status in SLE Patients and Healthy Controls. (A) The plasma level concentration of the IFN-α is plotted for SLE patients and for healthy controls. (B) The plasma concentration of IFN-α in SLE groups. (C) Dot plots showing IFN-α gene expression in SLE patients and in healthy controls. (D) The IFN-α gene expression from SLE patients. Dash lines in A, B indicate detection limit concentration of IFN-α (1 pg/ml). 1 star, 3 stars, 4 stars, and ns represents P<0.05, P<0.001, P<0.0001, and non-significant, respectively. P-values were measured utilizing the Mann–Whitney test.

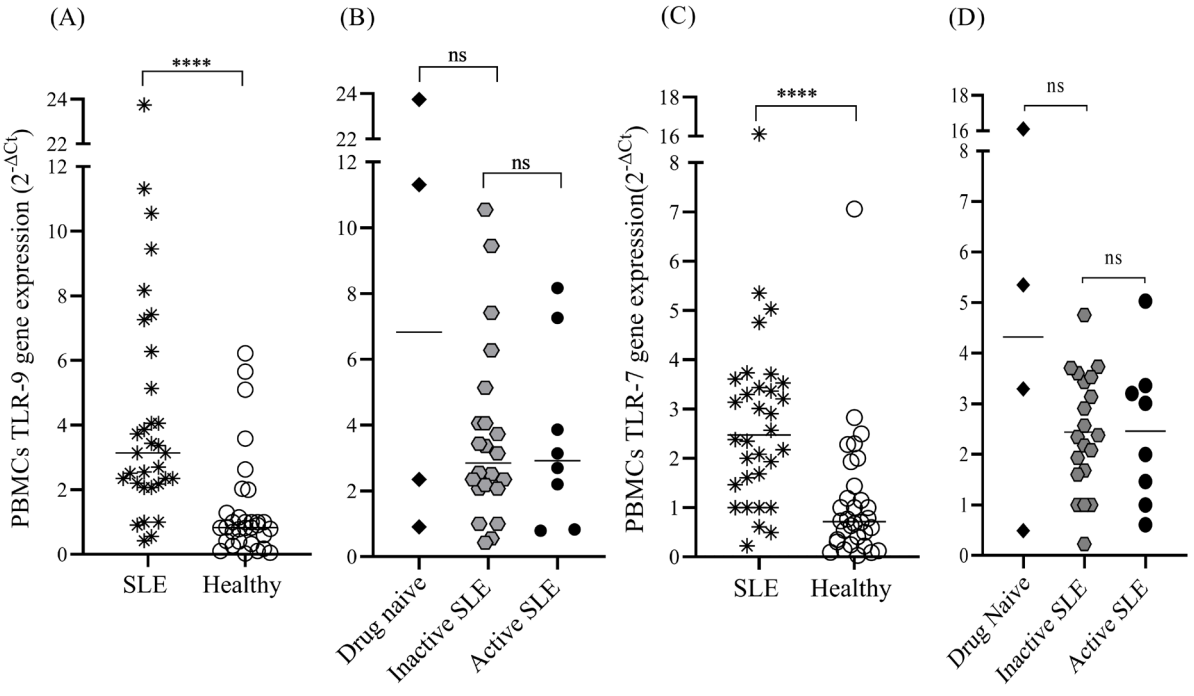

Figure 4. TLR7, and TLR9 Gene Expression in PBMCs. (A) Dot plots showing TLR9 gene expression in SLE patients and in healthy controls. (B) TLR9 gene expression in SLE groups. (C) Dot plots showing TLR7 gene expression in SLE patients and in healthy controls. (D) TLR7 gene expression in SLE groups. 4 stars and ns indicate P<0.0001 and non-significant, respectively. P values were specified applying the Mann-Whitney test.

(P>0.05) (Figure 3D).

TLR7 and 9 Gene Expression in PBMCs

SLE patients also expressed significantly higher TLR9 (median, range: 3.13, 0.42-23.75) than in healthy controls (0.82, 0.026-6.22) (P<0.0001) (Figure 4A). However, we found no significant difference when data from SLE patients were analyzed based on disease forms (Figure 4B). SLE patients also expressed significantly higher TLR7 (2.473, 0.22-16.11) than in healthy controls (0.71, 0.03-7.06) (P<0.0001) (Figure 4C) although great differences were not observed when data were analyzed based on disease forms (Figure 4D).

DISCUSSION

Given the feasible role of EBV infection in immune complex diseases such as SLE and the highlighted role of ongoing IFN-α production in the majority of patients, here we aim to assess the overall status of the EBV infection, TLR7, TLR9, and the IFN-α in SLE patients and in healthy controls. In the current study, comparison of SLE patients with healthy controls showed that (1) humoral immune responses to the EBNA1 (the anti-EBNA-1 IgG) increased in SLE patients; (2) the EBV viral loads in PBMCs rose in SLE patients; (3) the plasma level of the IFN-α was remarkably greater; (4) The gene expression of the IFN-α, TLR7, and TLR9 in PBMCs were significantly higher.

In agreement with previous studies (34-36), the anti-EBNA-1 IgG titer was considerably higher in SLE patients than in the controls. The Anti-EBNA-1IgG titers in patients with inactive SLE notably increased than in those with active form, which may be because the number of patients with inactive forms was more than in other groups (active or drug-naïve patients). Given the fact that SLE-specific autoantibodies such as dsDNA have the potential to cross-react with the EBNA-1 (37), the increased titer of the anti-EBNA-1 IgG might be, in part, because of the increased titer of anti-dsDNA in these patients. Unlike these findings, Anette H.

Draborg et al., found no major difference in anti-EBNA-1 IgG titer between SLE patients and healthy controls (38). Even one study reported a lower frequency of anti-EBNA-1 IgG in SLE patients than in the healthy controls (39).

Looking at the EBV DNA load, SLE patients showed significantly higher viral load than in the controls. Our findings are consistent with previous reports, which indicate the abnormal elevation of the EBV load and defective control of latent EBV infection in these patients (12, 40). Abnormal regulation of the EBV infection has been already reported in SLE patients. Gross et al., have shown high frequencies of the EBV-infected cells in the blood of SLE patients, which was associated with the occurrence of SLE disease flares. Moreover, they detect aberrant expression of viral lytic and latency genes in the patients' blood (41). Although fewer data are available in Iran with regards to the EBV status in SLE patients, Ahmad Piroozmand et al., have recently reported significantly higher EBV load in serum of SLE patients with active disease than in inactive SLE patients (P=0.003) (42).

Although the present study cannot rule out the causative role of the EBV infection in SLE disease, it further supports the relationship between the EBV infection and lupus, as has been previously highlighted (43). Several studies have found the unusual function of B cells in patients with SLE including alterations in blood B cell subset (44), abnormalities in the peripheral B cell compartment together with intensive germinal center activity (45), alteration in the B cell receptor (BcR) signaling thresholds (46), and aberrant early signal transduction events in B cells (47). Given the fact that B cells act as a main source of latency in the EBV infection, any changes in the state and the function of B cells could potentially affect the EBV infection and the overall status of the EBV infection in these patients. The immunoregulatory changes that can occur as a consequence of the disease may also compromise the host intrinsic resistance to virus infection in these patients (43). While SLE is mainly considered as a B cell-mediated disorder, SLE patients were also found to exert defective suppressor T cell responses by showing T cells being less capable to restrain the outgrowth o virus-infected B cells from in vitro–infected peripheral blood cells (48).

The plasma levels of the IFN-α were significantly higher in SLE patients than in the healthy controls. This study's findings are consistent with previous research (7, 49), although some studies could not find such a difference between SLE patients and healthy controls (50). Since the composition of pDCs subtypes may vary among patients and healthy individuals, excessive or prevention of the IFN-α production cannot be solely justified by cell count and may be explained by other factors (50). A suggestion has been also made that other cells including B cells can produce the IFN-α in these patients (51). The type and dosage of treatments in SLE patients can also affect the function of pDCs (52).

Elevated levels of TLR7, and TLR9 have been previously involved in the pathogenesis of lupus in both humans and animal models (53). In line with these findings, the mRNA levels of TLR7, and TLR9 were considerably greater in SLE patients than in healthy controls. Given the multiple effects of TLR activation during primary EBV infection that may favor viral latency or reactivation, it is unclear whether the overall effects of TLR stimulation would further complicate the associated immunopathological symptoms during primary EBV infection or exert beneficiary effects in these patients (54). Given the fact that the EBV infection establishes lifelong latency, the virus can act as a continuous source of chronic immune stimulation, which could trigger systemic autoimmune responses and was related to the rise in the production of lupus autoantibodies (55). Infection with the EBV appears to perpetuate the cycle of inflammation in SLE by modulating IFN production although it is not yet clear to what extent the elevated

IFN signatures observed in SLE patients are a response to the EBV stimulation (17). However, the changes in the EBV behavior may simply be associated with the defects in immune function, and deregulated EBV infection per se cannot be interpreted as a causative factor in SLE (41).

While studies are mainly limited to the blood status of the EBV, examining the EBV status in the lymphoid tissues would provide further insight in terms of the EBV pathogenesis in SLE. Moreover, a relatively small sample size particularly drug-naïve patients should be acknowledged when interpreting data.

CONCLUSION

Our results further highlight the potential role of the EBV infection and the IFN-α status in SLE patients although further studies should determine the worldwide footprint for the EBV infection in SLE patients by use of the immunological signature.

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Conflicts of Interest: None declared.

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