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ORIGINAL ARTICLE

Glucocorticoid-induced leucine zipper expression is associated with response to treatment and immunoregulation in systemic lupus erythematosus

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Abstract *Systemic lupus erythematosus* (SLE) is an autoimmune disorder in which cytokine balance is disturbed. *Glucocorticoids* (GCs) are shown to balance immune response by transcriptional regulation of glucocorticoid receptor target genes such as *Glucocorticoid-induced leucine zipper* (GILZ) which has been introduced as an endogenous anti-inflammatory mediator. In the present study, we assessed the expression of GILZ in association with interferon-γ (IFN-γ), interleukine-10 (IL-10), and B lymphocyte stimulator (BLyS) plasma levels in SLE patients. A total of 40 female patients (18 under treatment and 22 newly diagnosed) were recruited in this study. Real-time RT PCR was conducted to quantify the mRNA expression of GILZ. The plasma levels of IFN-γ, IL-10, and BLyS were evaluated using ELISA method. GILZ was overexpressed among under treatment SLE patients. The mRNA expression of GILZ was significantly correlated with Systemic Lupus Erythematosus Disease Activity Index (SLEDAI) score. IFN-γ and BLyS were downregulated in response to therapies with negative correlations to GILZ. Moreover, IL-10 was upregulated among treated patients. The levels of IFN-γ and BLyS were correlated with the severity of disease, while IL-10 was negatively correlated with SLEDAI score. GILZ could be introduced as one of the acting molecules in mediating the regulatory effects of GCs on producing pro- and anti-inflammatory cytokines in SLE.

Keywords B lymphocyte stimulator · Glucocorticoid-induced leucine zipper · Interferon-γ · Interleukin-10 · Systemic lupus erythematosus

Introduction

Systemic lupus erythematosus (SLE) is a heterogeneous autoimmune disorder with diverse clinical symptoms in which tolerance against self-antigens is lost [1]. The clinical manifestations may range from mild skin rashes to severe renal, central nervous system, or hematologic involvements [2]. Despite different proposed mechanisms, underlying cause is still unknown. The environmental or infectious factors in genetically susceptible individuals trigger the onset of disease and lead in acute to chronic immune activation [3].

Glucocorticoids (GCs) are commonly being used in the treatment of SLE and other autoimmune disorders [4]. The anti-inflammatory and immunosuppressive effects of GCs are complex and depend on the induction of anti-inflammatory proteins, as well as inhibition of signaling pathways such as NF-κB and AP-1. *Glucocorticoid-induced leucine zipper* (GILZ) is an early-response transcription regulator with physiologic role in the regulation of inflammation [5]. GILZ overexpression has been shown to inhibit the production of inflammatory cytokines such as IL-1β and TNF-α [6], chemokines [7], and adhesion molecules [8] in different cells of immune system. GILZ could mediate phenotypic characteristics that enhance differentiation of regulatory T cells and

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dampen of effector T cells [9, 10]. It could also redirect the maturation of human dendritic cells and prevent their antigen-specific T lymphocyte response [11, 12]. However, the underlying molecular mechanisms regarding the anti-inflammatory and immune suppressive properties of GILZ are not well elucidated in SLE.

B lymphocyte stimulator (BLyS) is a TNF cytokine family ligand which is determinant in B cell differentiation and autoantibody production. High levels of BLyS have been reported in different states of SLE [13]. The activation of immune response in SLE is mainly associated with an imbalance between inflammatory T helper cell subtypes (Th1 and Th17) and regulatory T cells (Treg) [14]. Although type I interferons with IFN-α as the dominant mediator are known as central to the pathogenesis of SLE [15], interferon-γ (IFN-γ) is also known as one of the important pro-inflammatory cytokines and the activator of inflammatory immune responses in several autoimmune disorders [16]. Some of the pathologic features of SLE may be associated with an IFN-γ-derived immune response [17, 18]. However, GCs could suppress the expression of IFN-γ and its downstream signaling pathways [19]. On the other hand, interleukine-10 (IL-10) is an anti-inflammatory cytokine which may inhibit the activity of Th1 and Th17 cells, while Th2 cells produce more IL-10 [20]. IL-10 is also known as a potent cofactor for the proliferation and survival of B cells [21].

Imbalanced production of pro-inflammatory cytokines and deregulated immune response could be associated with systemic inflammation and end organ damage in SLE [22]. In order to develop more efficient drugs and monitoring the response to current treatments in SLE, the mechanisms involved in sustained inflammation should be well understood. Here, we investigated the mRNA expression of GILZ as the acting mediator in GCs effect and the plasma levels of IFN-γ, IL-10, and BLyS in SLE patients in correlation with the disease activity.

Materials and methods

Patients and controls

A total of 40 female patients (18 under treatment and 22 newly diagnosed) fulfilling 4 out of 11 items of the revised American College of Rheumatology (ACR) criteria for SLE (either clinical manifestations or laboratory findings) [23] were enrolled in this study at Sayyad Shirazi educational hospital, rheumatology department, Golestan University of Medical Sciences, Gorgan, Iran. A written informed consent was taken from all patients following the Declaration of Helsinki [24]. All patients with an active infection, pregnancy, and/or history of other autoimmune disorders were excluded. The disease activity was calculated in SLE patients using Systemic Lupus Erythematosus Disease Activity Index (SLEDAI) by a specialist [25]. Twenty age- and sex-matched healthy donors were also recruited. This study was approved by the ethical committee of Golestan University of Medical Sciences. A volume of 5 ml whole blood using 3.8% sodium citrate as the anticoagulant was taken from all subjects, and plasma was separated and stored at −80 °C for the measurement of cytokines. Peripheral blood mononuclear cells (PBMCs) were also isolated using Ficoll-Paque (Baharafshan, Tehran, Iran) density-gradient centrifugation, as previously described [26]. The laboratory and clinical characteristics of SLE patients are illustrated in Table 1.

RNA isolation and reverse transcription real-time quantitative PCR analysis

Total RNA was isolated from PBMCs using Biozol (Bioer, China) according to the manufacturer's protocol. One microgram of total RNA was reverse transcribed to cDNA with random hexamer primers using Bioron cDNA synthesis kit (Bioron, Germany) and also treated with DNase I (Sinaclon,

Table 1 Clinical characteristics and laboratory parameters of patients with systemic lupus erythematosus in different groups and healthy donors

Charactristics	Under treatment ($N = 18$)	Newly diagnosed ($N = 22$)
Age[a]	34.00 ± 15.00	31.00 ± 18.00
SLEDAI[a]	4.00 ± 4.00	20.50 ± 24.00
Anti-dsDNA titer[a]	60.00 ± 53.00	172.00 ± *252.00*
WBC count[b]	5636.11 ± 409.32	4186.36 ± 243.45
ESR[a]	32.00 ± 38.00	49.00 ± 14.00
RF-IgG[a]	17.00 ± 41.00	51.00 ± 36.00
Hair loss[c]	3 (16.66%)	12 (54.54%)
Lupus nephritis[c]	2 (11.11%)	4 (18.18%)
Malar rash[c]	6 (33.33)	15 (68.18%)

WBC white blood cells, *ESR* erythrocyte sedimentation rate, *RF* rheumatoid factor, *SLEDAI* systemic lupus erythematosus disease activity index

[a] Data were demonstrated as medians ± IQR (interquartile range) for skewed variables

[b] Means ± SE (standard error) for normally distributed values

[c] Number (percentage) for categorical variables

Iran) to remove any genomic DNA contaminations. Real-time RT-PCR was performed with Bioer detection system (Bioer). PCR amplifications were performed using Bioron SYBR green qPCR master mix (Bioron). Eighteen seconds ribosomal RNA (18s rRNA) was used as a suitable internal control for gene expression normalization using the following primers: forward: 5′-CAGCCACCCGAGATTGAGCA-3′; reverse: 5′-TAGTAGCGACGGGCGGTGTG-3′. Gene specific primers for GILZ were as follows: forward: 5′- TCTGCTTG GAGGGGATGTGG-3′ and reverse: 5′-ACTT GTGGGGATTCGGGAGC-3′. All primers were designed and evaluated to span exon-exon junctions.

Quantification the plasma level of IFN-γ, IL-10, and BLyS using ELISA

The plasma level of IFN-γ and IL-10 in SLE patients and healthy donors were determined using commercially available ELISA kit (Biolegend, CA, USA) according to the manufacturer's instructions. Human BLyS Instant ELISA kit (eBioscience, USA) was also used to evaluate the plasma level of BLyS. Biotek ELISA reader ELX800 (Biotek, VT, USA) was used to obtain the optical density of each sample at the wavelength of 450 nm. All samples were assayed in triplicates, and the results were reported as picograms per milliliter (pg/mL). All tests were initially performed blind, and patients' information were not known to the individual conducting the assays.

Statistical analyses

In order to test the normal distribution of variables in each group, Shapiro-Wilk test was performed. Continuous data were demonstrated as means ± SE (standard error) for normally distributed values and medians ± IQR (interquartile range) for skewed variables. Categorical variables were shown as number (percentage). Statistical software SPSS 22.0 and Graphpad Prism 5.04 were used for data analyses and preparation of graphs. G* power 3.1 software [27] was also used to calculate the sample size and statistical power of the test on the basis of a previously conducted study [28]. One-way ANOVA with Tukey's post hoc test or nonparametric Kruskal-Wallis with Dunn-Bonferroni post hoc test were used to compare the means of multiple samples. Significant differences were also evaluated using independent samples *t* test or the equivalent nonparametric Mann-Whitney *U* test for two group comparisons. Fisher's exact test was used to determine the differences between two study groups with categorical variables. Spearman correlation study (two-tailed) was performed to evaluate the correlation between parameters. *P* values lower than 0.05 were considered as statistically significant.

Results

Comparison of clinical and laboratory findings between newly diagnosed and treated patients

Results from Kruskal-Wallis test revealed that the mean SLEDAI scores of newly diagnosed SLE patients were significantly higher than patients receiving treatment ($P = 0.002$) and healthy controls ($P < 0.0001$). Anti-dsDNA antibody titers were also significantly higher among newly diagnosed patients ($P < 0.0001$). We also showed that there were remarkable changes comparing white blood cell (WBC) counts ($P = 0.003$) and rheumatoid factor (RF) levels ($P = 0.008$) between two groups of SLE patients, while erythrocyte sedimentation rate (ESR) levels were not significantly different (*P* value = 0.066). The results of Fisher's exact test showed that incidences of malar rash ($P < 0.0001$) and hair loss ($P < 0.0001$) were significantly higher among newly diagnosed SLE patients. However, there was no significant difference ($P = 0.146$) between lupus nephritis occurrences in these two groups of patients. We also assessed the relevance between IFN-γ, IL-10, BLyS, and GILZ levels with the clinical and laboratory characteristics among patients. As summarized in Table 2, there were significant correlations between IFN-γ, BLyS, and anti-dsDNA antibody levels. IL-10 and GILZ levels were also negatively correlated with anti-dsDNA antibody.

Table 2 Correlation of clinical and laboratory charactristics with IFN-γ, IL-10, BLys, and GILZ expression levels

Characteristics	Anti-dsDNA titer (μM)		WBC count (per μL)		ESR		RF-IgG (μM)	
	r_s	*P* value	r_s	*P* value	r_s	*P* value	r_s	*P* value
IFN-γ	0.3329	0.0358	−0.0764	0.6393	−0.0539	0.7410	0.0698	0.6683
IL-10	−0.4670	0.0024	0.4128	0.0081	−0.1115	0.4932	−0.2769	0.0837
BLyS	0.3361	0.0340	−0.3244	0.0411	0.2121	0.1888	0.2140	0.1849
GILZ	−0.4005	0.0104	0.1912	0.2373	−0.2716	0.0900	−0.2693	0.0929

Two-tailed spearman correlation study was performed to evaluate the correlation between parameters. *P* values lower than 0.05 were considered as statistically significant

r_s Spearman correlation coefficient, *WBC* white blood cells, *ESR* erythrocyte sedimentation rate, *RF* rheumatoid factor

GILZ is overexpressed among under treatment SLE patients

Real-time RT-PCR results revealed that the mRNA expression of GILZ in the PBMCs of patients receiving treatments is significantly higher than newly diagnosed patients ($P < 0.0001$) and normal subjects ($P < 0.0001$). Moreover, there was no significant difference between normal subjects and newly diagnosed patients (Fig. 1).

Plasma level evaluation of IFN-γ, IL-10, and BLyS

The levels of IFN-γ, IL-10, and BLyS were also assessed in plasma using ELISA method. The level of IFN-γ was significantly higher among newly diagnosed SLE patients in comparison to healthy donors ($P < 0.0001$) and under treatment patients ($P < 0.0001$).There was no significant difference between IFN-γ plasma level among normal subjects and under treatment patients ($P = 0.197$) (Fig. 2a). The expression level of IL-10 was shown to be higher among treatment receiving SLE patients compared to newly diagnosed patients ($P < 0.0001$) and normal subjects ($P = 0.016$). Regarding the reduction of IL-10 plasma level in newly diagnosed patients, a significant change was observed ($P = 0.005$) in comparison to healthy controls (Fig. 2b). BLyS was also reported to be overexpressed in the plasma of newly diagnosed SLE patients in comparison to treatment receiving patients ($P < 0.0001$) and normal subjects ($P < 0.0001$). The differences between BLyS plasma level of normal subjects and treatment receiving patients were not noticeable ($P = 0.734$) (Fig. 2c).

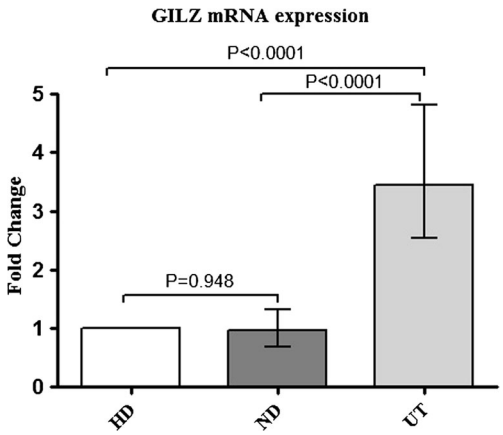

Fig. 1 GILZ mRNA expression in PBMCs of SLE patients (*UT* under treatment, *ND* newly diagnosed). Data are expressed as fold changes relatively to mRNA levels in healthy donors (HD), arbitrarily set at the value of 1. Data are presented as medians ± IQR (interquartile range). Significant differences are evaluated using nonparametric Kruskal-Wallis with Dunn-Bonferroni post hoc test. *P* values lower than 0.05 are considered statistically significant

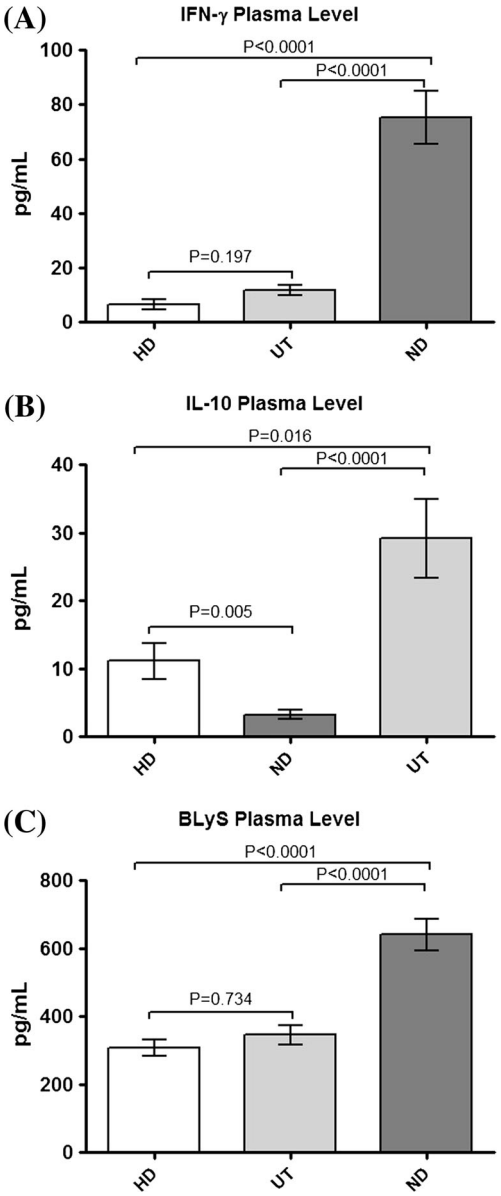

Fig. 2 The levels of IFN-γ (**a**), IL-10 (**b**), and BLyS (**c**) in the plasma of SLE patients. One-way ANOVA with Tukey's post hoc test or Kruskal-Wallis with Dunn-Bonferroni post hoc test were used to compare the means of multiple samples. All of the experiments were repeated in triplicates for each sample. Data of each bar demonstrates means ± SE for BLyS (normally distributed) and medians ± IQR for IL-10 and IFN-γ (skewed) variables. *P* values lower than 0.05 were considered as statistically significant. *SE* standard error, *IQR* interquartile range, *UT* under treatment, *ND* newly diagnosed, *HD* healthy donors

GILZ mRNA expression is correlated with SLEDAI score in SLE

Spearman correlation study was performed to evaluate the correlation between expression of GILZ in the PBMCs of SLE patients and SLEDAI scores. As shown in Fig. 3, there was a significant negative correlation between GILZ mRNA expression and SLEDAI score ($r_s = -0.6598$, $P < 0.0001$).

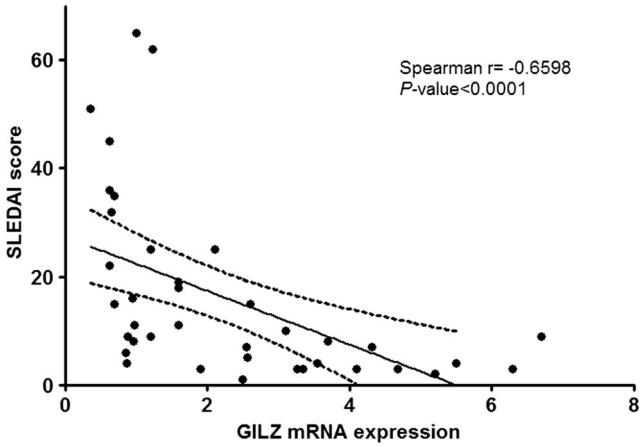

Fig. 3 Significant negative correlation between GILZ mRNA expression and SLEDAI score in SLE. (Spearman correlation coefficient (r_s) = −0.6598, $P < 0.0001$). Two-tailed spearman correlation study was performed to evaluate the correlation between GILZ expression and SLEDAI score. P values lower than 0.05 were considered as statistically significant

Correlation analyses between IFN-γ, IL-10, BLyS, and GILZ mRNA expression

To evaluate the relevance between IFN-γ, IL-10, BLyS, and GILZ, a two-tailed Spearman correlation study was carried out. As shown in Fig. 4a, there was a significant reverse correlation between IFN-γ plasma levels and GILZ expression (r_s = −0.6384, $P < 0.0001$). There was also a significant correlation between IL-10 plasma levels and GILZ mRNA expression (r_s = 0.4946, P = 0.0012) (Fig. 4b). As shown in Fig. 4c, there was a significant reverse correlation between BLyS plasma level and GILZ mRNA expression (r_s = −0.5437, P = 0.0003). Moreover, IFN-γ was significantly correlated with BLyS plasma level (r_s = 0.6537, $P < 0.0001$) (Fig. 4d). There was a significant reverse correlation between BLyS plasma level and IL-10 (r_s = −0.5235, P = 0.0005) (Fig. 4e). Similarly, a significant reverse correlation was also shown to be present between IFN-γ and IL-10 levels (r_s = −0.6147, $P < 0.0001$) (Fig. 4f).

SLEDAI score is correlated to IFN-γ, IL-10, and BLyS

We also evaluated the relevance between IFN-γ, IL-10, BLyS, and SLEDAI scores among SLE patients. There was a significant positive correlation between IFN-γ levels and SLEDAI (r_s = 0.8164, $P < 0.0001$) (Fig. 5a). A significant negative correlation was also observed between IL-10 levels and SLEDAI (r_s = −0.6055, $P < 0.0001$) (Fig. 5b). As shown in Fig. 5c, we showed that there was a significant positive correlation between BLyS level and SLEDAI scores (r_s = 0.5579, P = 0.0002).

Discussion

The overexpression of inflammatory cytokines could be associated with the imbalanced immune response during systemic inflammation [22]. SLE is an autoimmune disorder in which the immune response is not properly regulated [1]. The disturbed immune response may lead to sustained chronic inflammation and dysfunction of multiple organs [2, 3]. Cytokine imbalance is a common feature in systemic inflammatory disorders such as SLE which could be monitored to better find out the state of disease and response to treatments. Despite the harmful side effects that are mostly associated with the administration of GCs, this class of immunomodulatory drugs are still of the most common prescribed drugs against the majority of autoimmune diseases including SLE [4]. The anti-inflammatory and immunosuppressive effects of GCs are mostly mediated through interacting with the cytosolic glucocorticoid receptor (GR) and subsequent transcriptional regulation of target genes such as inhibiting the production of pro-inflammatory cytokines [29]. However, the molecular mechanisms responsible for the beneficial effects of GCs and mediator genes should be well understood. GILZ is a transcription regulator which could play an important role in immunoregulation through direct binding to the pro-inflammatory factors [5]. GILZ overexpression is also associated with the differentiation of regulatory T cells [9]. In order to monitor the possible changes in the expression of GILZ during treatment, we addressed the mRNA expression of GILZ in the PBMCs of newly diagnosed SLE patients in comparison to the patients receiving treatments and normal subjects. GILZ was significantly overexpressed in response to treatment among patients (Fig. 1) which denotes a mechanism of action for GCs therapy in systemic lupus which was in accordance with previous reports in other autoimmune diseases [30]. There was also a significant reverse correlation between GILZ expression and the disease severity (SLEDAI score) (Fig. 3). While the expression pattern of GILZ is associated with the state of disease, GILZ could be introduced as marker in assessing the disease activity and response to GC-based therapies in SLE.

Regarding the upregulation of GILZ in response to treatment, we also evaluated the levels of BLyS, IFN-γ, and IL-10 to deeply investigate the role of each cytokine among patients and in comparison to normal subjects. While the role of GILZ in cytokine modulation is to be elucidated as a master regulator or bystander, exploring the cytokine alterations in correlation with GILZ expression could be so helpful. BLyS is an inflammatory mediator which plays a remarkable role in B cell differentiation and is shown to be overexpressed in different states of SLE [13]. In the recent study, GILZ has been introduced as a non-redundant regulator of B cell activity with important potential clinical implications in SLE. Jones et al. supposed that GILZ was reduced among B cells of patients

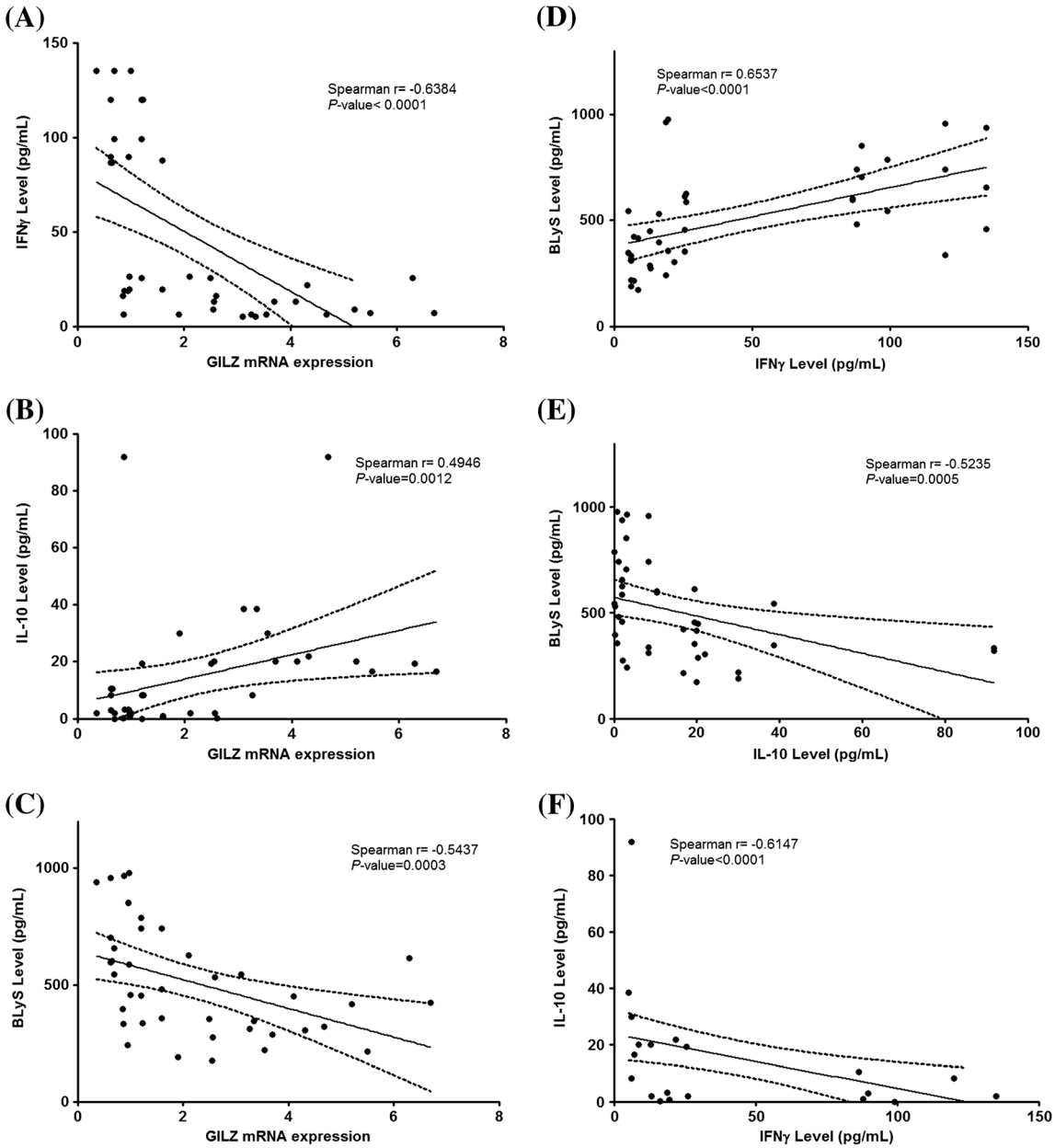

Fig. 4 Correlation of GILZ mRNA expression with IFN-γ, IL-10, and BLys plasma levels. Two-tailed spearman correlation study was performed. **a** Relationship between GILZ mRNA expression and IFN-γ level. GILZ is negatively correlated with IFN-γ (r_s = −0.6384, P < 0.0001). **b** Relationship between GILZ mRNA expression and IL-10 plasma level. GILZ is positively correlated with IL-10 (r_s = 0.4946, P = 0.0012). **c** Relationship between GILZ mRNA expression and BLyS plasma level. GILZ is negatively correlated with BLyS (r_s = −0.5437, P = 0.0003). **d** Relevance between IFN-γ and BLyS plasma levels. IFN-γ is positively correlated with BLyS (r_s = −0.6384, P < 0.0001). **e** Relationship between BLyS and IL-10 plasma levels. BLyS is negatively correlated with IL-10 (r_s = −0.5235, P = 0.0005). **f** Relevance between IFN-γ level and BLyS. BLyS is negatively correlated with IFN-γ (r_s = −0.6147, P < 0.0001). P values lower than 0.05 were considered as statistically significant

with SLE and lupus-prone mice, and they also observed that impaired induction of GILZ in GC-receiving SLE patients was associated with increased disease activity [31]. BLyS was downregulated in the plasma of under-treatment SLE patients (Fig. 2c). It was also shown to be negatively correlated with the mRNA expression of GILZ (Fig. 4c). This could bring further evidence to the role of GILZ as a B cell regulator through altering the expression of BLyS [13, 32]. IFN-γ plays an important role in the pathogenesis of SLE through sustained inflammatory immune response [33]. Although GCs could regulate the expression of IFN-γ [19], the regulated target genes and downstream signaling pathways should be well understood. Here, we showed that IFN-γ was produced in higher levels among newly diagnosed SLE patients (Fig. 2a). While there is a reverse correlation between the levels of IFN-γ and GILZ (Fig. 4a), GILZ could be introduced

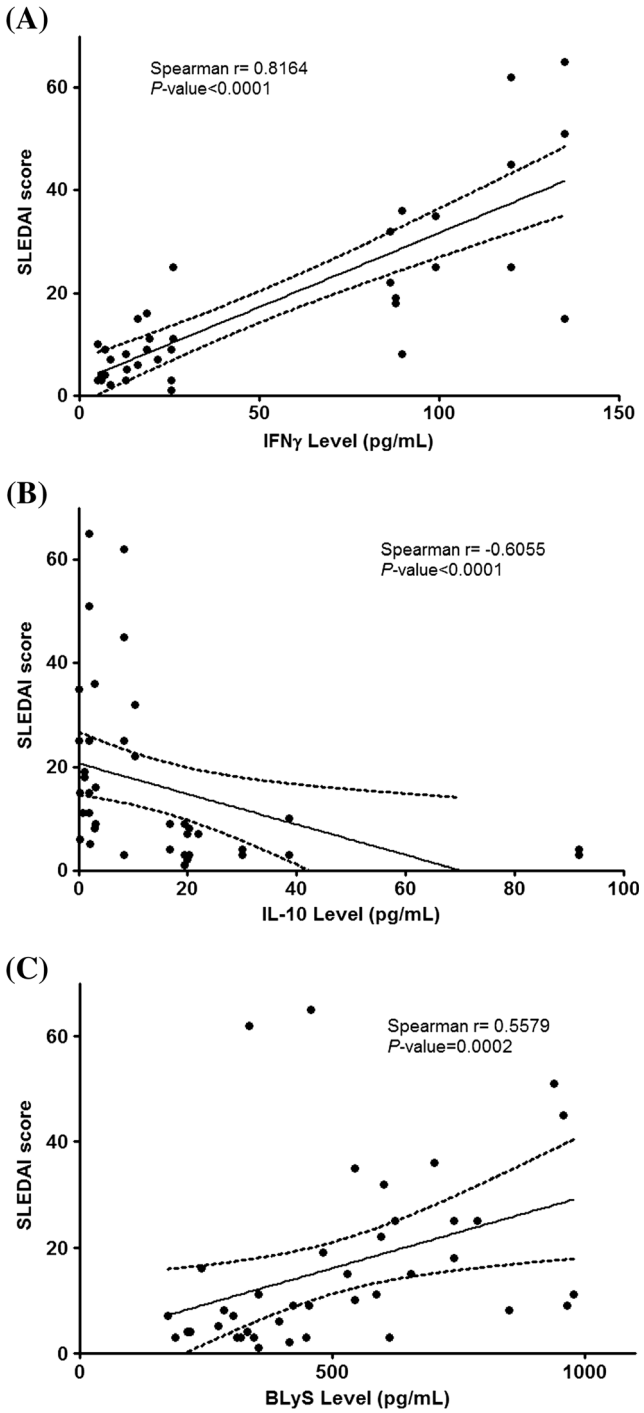

Fig. 5 Correlation of SLEDAI score with IFN-γ, IL-10, and BLys plasma levels. Two-tailed spearman correlation study was performed. **a** Relationship between SLEDAI score and IFN-γ level. SLEDAI is positively correlated with IFN-γ (r_s = 0.8164, P < 0.0001). **b** Relationship between SLEDAI and IL-10 plasma level. SLEDAI score is negatively correlated with IL-10 (r_s = −0.6055, P < 0.0001). **c** Relationship between SLEDAI score and BLyS plasma level. SLEDAI is positively correlated with BLyS (r_s = 0.5579, P = 0.0002). P values lower than 0.05 were considered as statistically significant

as a mediator in suppressing IFN-γ and IFN-γ-derived immune responses. A significant positive correlation was also shown between IFN-γ and BLyS (Fig. 4d). The excessive production of IFN-γ has been contributed to BLyS levels in a previous study [34] which could introduce BLyS as a molecule involved in the immunopathogenesis of SLE. As also reported previously [35], IL-10 was overexpressed in response to therapies among SLE patients (Fig. 2b) and was correlated with the GILZ expression (Fig. 4b). IL-10 was negatively correlated with BLyS and IFN-γ inflammatory mediators (Fig. 4e, f). Thus, it could exert the anti-inflammatory roles through regulating T cell responses and confining organ damages [20, 32].

Conclusions The cytokine profile balance is disturbed in autoimmune disorders including SLE. Administration of GCs has beneficial effects through transcriptional regulation of glucocorticoid receptor target genes such as GILZ which is overexpressed in response to routine therapies. GILZ could be introduced as a determinant of disease activity among SLE patients which is negatively correlated with the SLEDAI. The newly diagnosed SLE patients show higher levels of BLyS and IFN-γ and lower levels of IL-10 which denotes the overexpression of pro-inflammatory cytokines. Successful treatment strategies not only have dampened the inflammation but also have been associated with the overexpression of GILZ. Altogether, the effects of GCs on the regulation of pro- and anti-inflammatory cytokines in treated SLE are probably mediated by GILZ. However, supplementary clinical and laboratory experiments are needed to be performed.

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Compliance with ethical standards

Disclosures None.

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