Perforin level in CD4 T cells from patients with systemic lupus erythematosus

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Running heading: perforin expression in $CD4^+$ T cells

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Summary

$CD4^+$ T cells from patients with systemic lupus erythematosus (SLE) exhibit increased expression of various proteins contributing to defective function of $CD4^+$ T cells.

We evaluated the transcript and protein levels of perforin (PRF1) in $CD4^+$ T cells from SLE patients (n=41) and healthy individuals (n=34). The $CD4^+$ T cells were obtained by a positive biomagnetic separation system. The amounts of mRNA were determined by reverse transcription and real-time quantitative PCR. The protein levels in the $CD4^+$ T cells were evaluated by western blotting analysis. We observed significantly higher levels of PRF1 protein ($p= 0.013$) in SLE $CD4^+$ T cells than in controls. There was no significant increase in PRF1 transcript levels ($p=0.908$) in $CD4^+$ T cells from SLE patients as compared to healthy individuals. Moreover, we did not observe a correlation between PRF1 transcript and protein levels in SLE $CD4^+$ T cells and disease activity expressed by the SLEDAI scale.

We confirmed previous observations that demonstrated higher levels of PRF1 protein in $CD4^+$ T cells from SLE patients. However, we did not find a correlation between PRF1 transcripts and proteins in $CD4^+$ T cells and SLE disease activity.

Introduction

Systemic lupus erythematosus (SLE) is a progressive, systemic autoimmune disease with multi-organ engagement, characterized by an immune response directed against self antigens [1]. The SLE target tissues include skin, kidneys, joints, and the central nervous system [1]. Exposure to certain chemicals, drugs, food, and infectious factors also contributes to SLE incidence [2, 3]. Moreover, there is an evident genetic background in the incidence of this autoimmune disorder [4-6].

Three major processes are considered in the initiation, development and clinical manifestation of SLE; they include an increase in the plasma concentration of nuclear auto-antigens, T cell-dependent stimulation of B cells for the biosynthesis of antinuclear antibodies (Ab), and organ damage mediated by anti-double stranded DNA Ab and immune complexes [7-9].

Increased apoptosis and reduced removal of apoptotic cells lead to the formation of necrotic cells, which are source of nuclear antigens for biosynthesis of Ab [10, 11]. Cytotoxic T lymphocytes involved in killing other host cells may be significant producers of preferential and selective autoantigens [12]. T cells from lupus patients may kill autologous monocytes/macrophages, and may thus contribute to both increase of the amount of antigenic apoptotic material and the reduction of its clearance [13, 14].

It has been concluded that defective helper functions of $CD4^+$ T cells can contribute to improper activation of B cells and autoantibody production [15-18]. Despite $CD4^+$ T cells function mainly as helper cells, a, subpopulation of these cells also functions in an effector capacity by carrying out cytotoxicity in a peptide-specific and MHC class II-restricted mode [19]. The latter function of $CD4^+$ T cells mainly depends on granzyme B and perforin-1 (PRF1) exocytosis rather than on CD95-ligand binding to CD95 [19]. An increased presence of cytotoxic $CD4^+$ T cells has been associated with vascular damage and incidence of various

autoimmune diseases, including SLE [20-24]. The overexpression of PRF1 in both $CD4^+$ T and $CD8^+$ T cells from peripheral mononuclear cells (PBMC), and its role in SLE pathogenesis has been demonstrated [24, 25]. Kaplan *et al.* (2004), employing staining of PBMC and flow cytometry analysis, showed a significant correlation between percentage of $PRF1^+$ $CD4^+$ T cells in PBMC and disease activity in SLE patients [24].

We decided to examine mRNA and protein levels in whole $CD4^+$ T cells from a larger group of SLE patients (n=41) and healthy individuals (n=34) than those demonstrated by Kaplan *et a.* [24]. Whole $CD4^+$ T cells from whole peripheral blood were isolated by positive biomagnetic separation. Isolated mRNA and proteins from $CD4^+$ T cells were used for reverse transcription, real-time quantitative PCR and western blotting analysis. Subsequently we determined whether there exist correlations between increased PRF1 transcript and protein levels in $CD4^+$ T cells and SLE disease activity.

Materials and methods

Patients and controls

Forty-one consecutive SLE patients seen between September 2006 and October 2008 at the Institute of Rheumatology in Warsaw and Department of Rheumatology and Clinical Immunology at the Poznań University of Medical Sciences, Poland were included in the present study (Table 1). Patients fulfilled at least 4 of the American College of Rheumatology 1982 revised criteria for SLE [26, 27]. Disease activity for the SLE patients was assessed using the SLEDAI scale (Table 1) [28]. Twenty-four patients were treated by prednisone. Seventeen patients were receiving methylprednisone, and ten patients were also receiving hydroxychloroquine. In order to reduce the effect of corticosteroid medication on our results, patients who were on prednisone or methylprednisone were asked not to take this medication for at least 24 h before drawing blood. Clinical manifestations of SLE in the patient group

included central nervous system (22%), vascular (20%), renal (42%), musculoskeletal (29%), serosal (20%), dermal (34%), immunologic (85%), febrile (8%), and hematologic (29%) components.

Both patients and control groups were of Polish Caucasian origin. The control group included thirty-four healthy individuals (32 women and 2 men). The mean age of healthy individuals was 36.6 ±9.6 years. The protocol of the investigation was approved by the Local Ethical Committee of Poznań University of Medical Sciences. Written informed consent was signed by all participating individuals.

Antibodies

Rabbit polyclonal anti- PRF1 Ab (H-315), goat anti-rabbit horseradish peroxidase (HRP)-conjugated Ab and anti-actin HRP-conjugated Ab (clone I-19) were provided by Santa Cruz Biotechnology (Santa Cruz, CA).

$CD4^+$ T cell isolation

The $CD4^+$ cells were isolated from a 10 ml whole peripheral blood sample taken from each patient, employing the positive biomagnetic separation technique using DETACHaBEADs ® M-450 CD4 from Dynal Biotech (Lake Success, NY) (29). The flow cytometry analysis (n=29) indicated 95.6 % ± 1.4 purity of $CD4^+$ T cells.

Real-time quantitative PCR (RQ-PCR) analysis of PRF1transcript levels in $CD4^+$ T cells

Total RNA was isolated according to the method of Chomczyński and Sacchi [29]. RNA samples were treated with DNase I, and 1 μg RNA was reverse-transcribed into cDNA using oligo-dT primers. RQ-PCR was conducted in a Light Cycler real-time PCR detection system from Roche Diagnostics GmbH, (Mannheim, Germany) using SYBR® Green I as detection dye. Target cDNA was quantified using relative quantification method with a calibrator. The calibrator was prepared as a cDNA mix from all SLE and control samples and

consecutive dilutions were used to create a standard curve as described in Relative Quantification Manual Roche Diagnostics GmbH, (Mannheim, Germany). For amplification, 2 µl of cDNA solution was added to 18 µl of QuantiTect® SYBER® Green PCR Master Mix QIAGEN GmbH (Hilden, Germany) and primers. The quantity of PRF1 transcript in each sample was standardized by human mitochondrial ribosomal protein L19 (hMRPL19).

The PRF1 cDNA 170 bp amplicon was amplified employing the pair of primers: 5'CACCCTCTGTGAAAATGCCCTAC3' (forward) and 5'TCCAGTCGTTGCGGATGCTAC3' (reverse).

The hMRPL19 cDNA 171bp amplicon was amplified using primers:

5'ACTTTATAATCCTCGGGTC 3' (forward) and 5'ACTTTCAGCTCATTAACAG 3' (reverse). These PRF1 and hMRPL19 primers were designed based on sequences ENST00000373209 and ENST00000393909 respectively, located in Ensembl Genome Browser (www.ensembl.org). The PRF1mRNA levels were expressed as multiples of these cDNA concentrations in the calibrator.

Western blot analysis of PRF1protein contents in $CD4^+$ T cells.

$CD4^+$ lymphocytes were lysed in lysis buffer, and ten micrograms of protein was resuspended in sample buffer. The proteins were separated on 10% Tris-glycine gel using sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to PVDF membrane. Immunodetection was performed with rabbit polyclonal anti-PRF1, and HRP-conjugated goat anti-rabbit Ab. The membranes were reblotted with anti-actin HRP-conjugated Ab to equalize protein loading of the lanes. To detect the examined protein from the same blot membrane we used stripping buffer. Bands were revealed using SuperSignal West Femto Maximum Sensitivity Substrate Pierce (Rockford, IL). The quantities of western blot-detected PRF1and β-actin proteins were determined based on the band optical density.

The band densitometry readings were normalized to β–actin loading control to calculate the PRF1–to β-actin optical density ratio.

Statistical analysis

Statistical analysis was conducted using StatSoft, Inc. (2001). STATISTICA (data analysis software system), version 6. www.statsoft.com. The significance of differences between groups was determined using nonparametric Mann-Whitney test. Correlations between PRF1expression and disease activity as defined by the SLEDAI scale were assessed by Spearman analysis.

Results

PRF1 transcript and protein levels in $CD4^+$ T cells from SLE patients and controls

To compare PRF1 transcript and protein levels in $CD4^+$ T cells from SLE patients and controls, we used RQ-PCR and western blotting analysis, respectively. There was no significant increase in PRF1 transcript level in SLE $CD4^+$ T cells as compared to controls ($p=0.908$) (Figure 1A and Table 2). However, we observed significantly higher content of PRF1 protein in $CD4^+$ T cells from SLE patients as compared to healthy individuals ($p= 0.013$) (Figures 1B, C, and Table 2). There was no correlation between PRF1 transcript and protein levels in SLE $CD4^+$ T cells and disease activity defined by the SLEDAI scale (Figures 2A and 2B). We also did not find a relationship between patients' age, disease duration, drug treatment, and type of individual organ manifestation with PRF1 transcript and protein levels in SLE $CD4^+$ T cells (data not shown).

Discussion

It has previously been demonstrated that there is an increased expression many proteins responsible for defective function of $CD4^+$ T cells [15,16, 18]. These proteins include signal molecules, transcription factors, components of TCR/CD3 complex, costimulatory proteins, cytokines and molecules involved in the cytotoxic response [15,16, 18].

PRF1 protein, with molecular mass of 60 to 75 kD, is a pore-forming molecule presented in cytotoxic lymphocytes, which execute immune mediated cell lysis [30]. The *PRF1* gene is expressed primarily in NK cells and effector $CD8^+$ T cells [31, 32]. *PRF1* is also expressed in a subpopulation of cytotoxic $CD4^+$ T cells, which may combat various pathogens (33-39). An increased number of cytotoxic $CD4^+$ T cells has been observed in patients with multiple sclerosis, rheumatoid arthritis, inflammatory bowel disease, and acute coronary syndromes [20-23].

Lu *et al* (2003) demonstrated that DNA methylation and chromatin structure regulate *PRF1* expression in T cells [40]. Moreover, Kaplan *et al.* (2004) showed that DNA methylation inhibitors increased *PRF1* expression in $CD4^+$ T cells. This increase in *PRF1* transcription in $CD4^+$ T cells was due to demethylation of a DNA region, which is hypomethylated in primary $CD8^+$ T cells expressing *PRF1* [24]. Kaplan *et al.* (2004) also indicated that increased *PRF1* transcription in $CD4^+$ T cells from SLE patients was related to demethylation of this DNA region [24]. Recently, Luo *et al.* (2009) disclosed that DNA demethylation at the *PRF1* locus is responsible for *PRF1* overexpression in $CD4^+$ T cells from patients with subacute cutaneous lupus erythematosus [41].

We observed significantly higher levels of PRF1 protein in SLE $CD4^+$ T cells than in healthy individuals. Our findings are consistent with Kaplan *et al.* (2004), who demonstrated a significant increase in PRF1 protein levels in SLE patients [24]. They also indicated that

aberrant PRF1 protein levels in SLE $CD4^+$ T cells may contribute to killing of autologous monocytes [24].

We also found higher PRF1 transcript levels in SLE $CD4^+$ T cells than in controls, but this difference was not statistically significant and did not correlate with disease activity. Although PRF1 protein level was higher in SLE $CD4^+$ T cells than in controls, it also did not correlate with disease activity. Our findings suggest that an increased PRF1 protein content in SLE $CD4^+$ T cells does not perpetuate disease activity in our investigated group.

Kaplan *et al.* (2004) observed that $CD4^+$ T cells from patients with active, but not inactive, lupus exhibited increased PRF1 transcript levels in $CD4^+$ T cells [24]. Using flow cytometry analysis, they also demonstrated that the increase in PRF1 protein contents in SLE $CD4^+$ T cells was correlated to disease activity expressed by the SLEDAI scale [29]. These discrepancies between our observations and those of Kaplan *et al.* may result from the application of different methods in the determination of PRF1 protein levels in $CD4^+$ T cells to establish correlation with disease activity.

The cytotoxic/effector $CD4^+$ T cells make up a small amount of the whole circulating $CD4^+$ T population [42]. These cells constitute the subset of $CD45R0^+CD27^-$ activated $CD4^+$ T cells, which are formed during differentiation of naive $CD45RA^+CCR7^+$ T cells [42, 43]. Therefore, our analysis, conducted on the whole $CD4^+$ T cell population after immune magnetic enrichment, may underestimate PRF1 transcript and protein levels in cytotoxic/effector $CD4^+$ T cells. This may be responsible for the discrepancies observed between our results and those of Kaplan *et al.* [24]. These differences can also be due to varied genetics factors associated with the distinct racial structure of the examined groups or/and an exposure to disparate environmental factors contributing to SLE in these populations [3,4].

Our study confirmed previous observations that demonstrated increased PRF1 protein contents in $CD4^+$ T cells from SLE patients. However, we did not find a correlation between PRF1 protein and transcript levels in $CD4^+$ T cells and SLE disease activity. Therefore our results require verification in groups of SLE patients in other cohorts.

Acknowledgements

Supported by grant No. N N402 2424 33 from Polish Ministry of Scientific Research and Information Technology. We would like to acknowledge Dr. Margarita Lianeri for her assistance.

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FIGURE LEGENDS

Fig.1. PRF1 transcript (**A**) and protein (**B**) levels and representative picture of western blot analysis (**C**) in $CD4^+$ T cells from SLE patients and controls.

The $CD4^+$ T cells were isolated from peripheral blood by positive biomagnetic separation technique, and were immediately used for RNA and protein isolation. Total RNA was reverse-transcribed, and cDNAs were investigated by RQ-PCR relative quantification analysis. The PRF1 mRNA levels were corrected to the amount of hMRLP19. The amounts of PRF1 mRNA were expressed as the natural logarithm of multiples of these cDNA copies in the calibrator. Proteins were separated using SDS-PAGE, transferred, and the membranes were immunoblotted respectively with primary anti-PRF1 Ab and secondary Ab. The membranes were reblotted with anti-β-actin HRP-conjugated Ab. The amount of western blot-detected PRF1 proteins was presented as the natural logarithm of PRF1–to β-actin band optical density ratio. (○) and (●) represent transcript levels in controls and SLE patients, respectively. *Median (range), ND-non detected.

Fig. 2. The correlation between PRF1 transcript (**A**) and protein (**B**) in $CD4^+$ T cells and clinical activity of SLE scored in the SLEDAI scale.

The $CD4^+$ T cells were used for RNA and protein isolation. Total RNA was reverse-transcribed into cDNAs and investigated by RQ-PCR relative quantification analysis. PRF1 mRNA levels were corrected to hMRLP19 levels. The quantities of PRF1 mRNA were expressed as the natural logarithm of multiples of these cDNA copies in the calibrator. The $CD4^+$ T cell proteins were separated using SDS-PAGE, transferred, and the membranes were immunoblotted respectively with primary anti-PRF1 Ab and secondary Ab. The membranes were reblotted with anti-β-actin HRP-conjugated Ab. The amount of western blot-detected PRF1 was presented as the natural logarithm of PRF1–to β-actin band optical densities ratio. r–Spearman correlation coefficient.

Table 1. Demographic, and disease activity of SLE patients

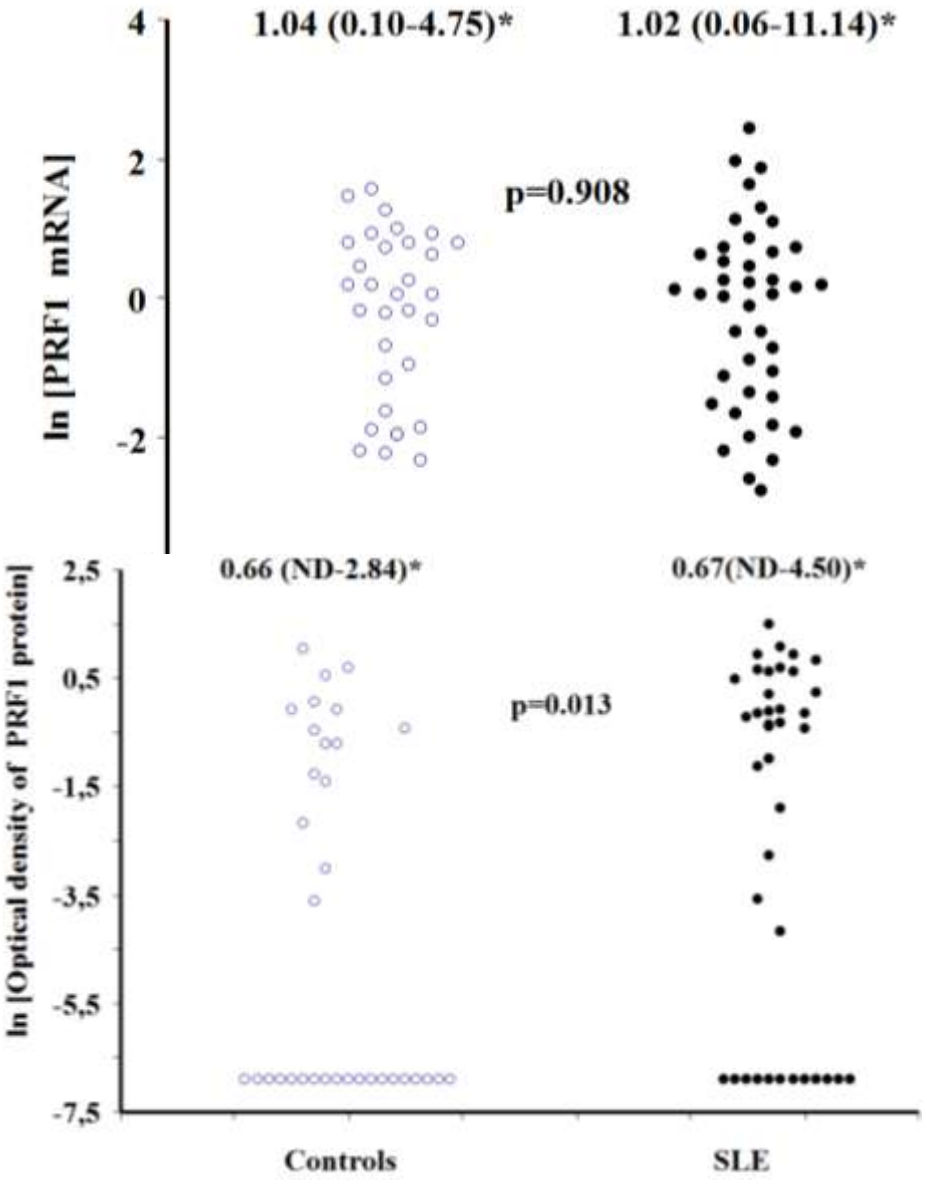
Patients Number	41
Sex (Female/Male)	39/2
Mean (± SD) of age at diagnosis (years)	36.2 ± 13.3
SLEDAI scores	Patients number
0-5	13
6-10	11
11-15	4
>16	13

Table 2. PRF1 transcript and protein levels in $CD4^+$ T cells from SLE patients and controls

	SLE		Controls		
	Median (range)	Mean (± SD)	Median (range)	Mean (±SD)	p
PRF1	1.02 (0.06-11.14)[a] 0.67 (ND-4.50)[b]	1.66 ± 2.23[a] 0.87 ± 1.04[b]	1.04 (0.10-4.75)[a] 0.66 (ND-2.84)[b]	1.34 ± 1.24[a] 0.37± 0.67 [b]	0.908[a] 0.013[b]

[a]The target mRNA levels were corrected to the amount of hMRLP19 cDNA and expressed as multiplicity of these cDNA copies in calibrator. [b]The amount of western blot detected proteins was presented as the PRF1 to β-actin band optical density ratio. [c]Mann-Whitney test, ND-non detected

A


1.04 (0.10-4.75)*
1.02 (0.06-11.14)*
ln [PRF1 mRNA]
4
2
0
-2
p=0.908

B

0.66 (ND-2.84)*
0.67(ND-4.50)*
ln [Optical density of PRF1 protein]
2,5
0,5
-1,5
-3,5
-5,5
-7,5
p=0.013
Controls
SLE

C

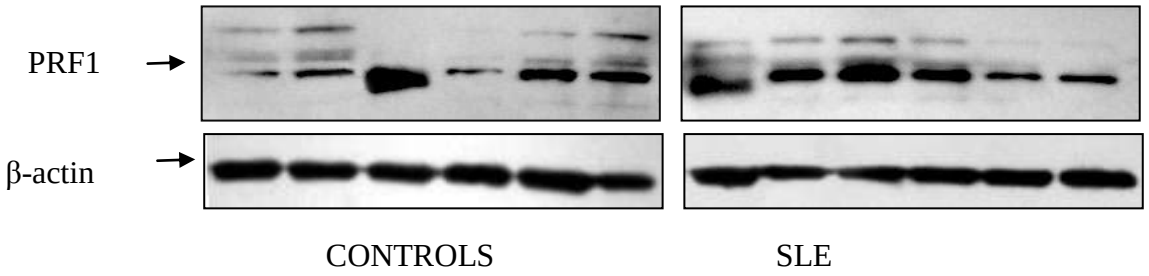
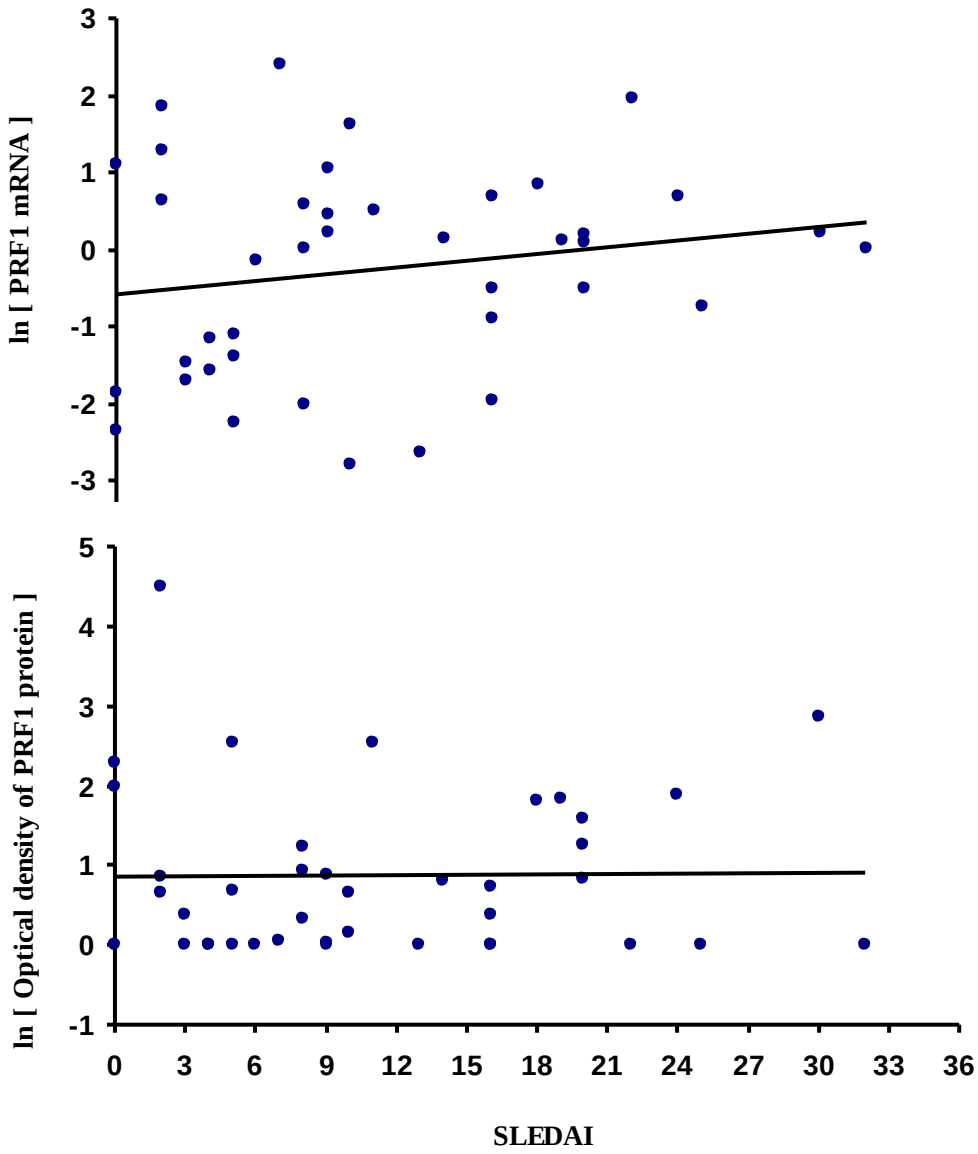


Fig. 1

A


ln [PRF1 mRNA]
3
2
1
0
-1
-2
-3

B

ln [Optical density of PRF1 protein]
5
4
3
2
1
0
-1
0
3
6
9
12
15
18
21
24
27
30
33
36
SLEDAI

Fig. 2