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ORIGINAL ARTICLE

Prevalence of ZAP-70, LAT, SLP-76, and DNA methyltransferase 1 expression in CD4$^+$ T cells of patients with systemic lupus erythematosus

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Abstract T cells from systemic lupus erythematosus (SLE) patients exhibit defective function of CD4$^+$ T cells that can be responsible for improper activation of B cells and antibody biosynthesis against host antigens. We compared the level of ZAP-70, LAT, and SLP-76, transcripts and proteins in CD4$^+$ T cells from SLE patients (n=22) and healthy individuals (n=15). We also determined DNA methyltransferase 1 (DNMT1) protein content in CD4$^+$ T cells of SLE patients. The CD4$^+$ T cells were isolated by positive biomagnetic separation technique. The quantitative analysis of messenger RNA (mRNA) was performed by reverse transcription and real-time quantitative polymerase chain reaction (RQ-PCR) SYBR Green I system. The protein level in the CD4$^+$ T cells was determined by Western blotting analysis. We found that the LAT protein level was significantly higher in SLE CD4$^+$ T cells than in controls (P=0.006). Western blot analysis revealed that ZAP-70 protein content in SLE CD4$^+$ T cells may be reciprocally correlated with disease activity expressed in SLEDAI scale (R=−0.623, P=0.002) or number of affected organ systems (R=−0.549, P=0.008). We also observed reciprocal correlation between DNMT1 protein content in CD4$^+$ T cells and disease activity scored with SLEDAI scale (R=−0.779, P=0.001) or number of affected organ systems (R=−0.617, P=0.019), respectively. Our findings might indicate that LAT, ZAP-70, and DNMT1 protein levels in CD4$^+$ T cells can be associated with SLE disease.

Keywords CD4$^+$ T cells · Signaling molecules · Systemic lupus erythematosus

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Introduction

Systemic lupus erythematosus (SLE) is a complex autoimmune disorder whose development is associated with various genetic and environmental factors [1–4]. SLE is characterized by abundant production of autoantibodies directed against self-antigens and formation of immunocomplexes that affect multiple organs [2, 5–7]. T cells from SLE patients exhibit various aberrations, which encompass skewed cytokine production, reduction in cytotoxic T cell function, and enhancement of humoral response [2, 8, 9]. The T cell receptor/CD3 (TCR/CD3) complex plays an elementary role in antigen recognition and the development of immune response. Stimulation of T cells is initiated by interaction of the TCR with an antigen bound to the major histocompatibility complex located on the surface of an antigen-presenting cell [10]. This interaction of T helper CD4$^+$ lymphocyte with B cells during antigen presentation is necessary for B cell differentiation into plasma cells and antibody production [2, 11]. The binding of the TCR with a specific antigen results in activation of Lck or Fyn non-receptor tyrosine kinases [10]. Activation of these kinases causes phosphorylation of the cytoplasmic domain of the immunoreceptor tyrosine-based activation motif of CD3-zeta (ζ) and ZAP-70 kinase, mainly functioning in naive T cells [8, 10, 12]. The activated ZAP-70 kinase phosphorylates linker/adapter molecules, including linker of activation of T cells (LAT) and Src homology 2 domain-containing leukocyte

protein of 76 kDa (SLP-76) [10, 13]. Phosphorylation of linker/adapter molecules triggers many biochemical intracellular events resulting in second messengers, transcription factor activation, and the expression of genes encoding proteins contributing to immune response [10, 14].

It has been believed that defective function of $CD4^+$ T cells can be responsible for improper activation of B cells and antibody biosynthesis against host antigens [2, 5, 8, 9]. The $CD4^+$ T cells from patients with SLE, compared to T cells from healthy individuals, exhibit numerous alterations in signal transduction and changed expression pattern of signal transducing molecules from TCR to nucleus [15]. The epigenetic aberration in regulatory DNA sequences may also be responsible for changes in expression of T cell proteins and the emergence of abnormalities in the immune system [16–19]. T cells from SLE patients display low expression of DNA methyltransferase 1 (DNMT1), which methylates cytosine in cytosine and guanine dinucleotides in new biosynthesized DNA strand during DNA replication [17, 18, 20].

We compared levels of transcript and protein of ZAP-70, LAT, and SLP-76, in $CD4^+$ T cells from SLE patients and healthy individuals. We also determined correlation between DNMT1 protein content in $CD4^+$ T cells and SLE disease activity scored in Systemic Lupus Erythematosus Disease Activity Index (SLEDAI) [21].

Materials and methods

Patients and controls

Twenty-two patients (22 women) fulfilling the American College of Rheumatology Classification (ACRC) criteria for SLE [6, 7] were chosen in a serial manner for investigation at the Institute of Rheumatology in Warsaw, Poland (Table 1). Seven, four, five, three, and two patients were, respectively, receiving prednisone in dosage 10, 15, 20, 30, and 40 mg per day, while six of these patients were also receiving hydroxychloroquine, and one was taking no medication. To reduce the effect of corticosteroid medication on our results, patients who were on prednisone were asked not to take this medication for at least 24 h before drawing blood. The protocol of the study was approved by the Local Ethical Committee of Poznañ University of Medical Sciences. Written informed consent was obtained from all participating subjects. Activity for the SLE patients was assessed using SLEDAI scale [21]. The control group included 15 healthy individuals (15 women). The age range of healthy individuals was 20–58 years with a median of 32 years.

Table 1 Demographic, organ involvement, and immunological findings of SLE patients

Features	
Number	22
Sex (female only)	22
Median of age at diagnosis (years)	37 (range 25–67)
Central nervous system*	2/22
Vascular*	0/22
Renal*	7/22
Musculoskeletal*	6/22
Serosal*	0/22
Dermal*	1/22
Immunologic*	11/22
Constitutional (fever)*	0/22
Hematologic*	4/22
Median of SLEDAI scores	5 (range 0–16)

*As defined by SLEDAI score index [22]

Antibodies

Goat anti-ZAP-70 Ab (C-20), goat anti-LAT Ab (Q-20), goat anti-SLP-76 Ab (C-20), goat anti-DNMT1 Ab (C-17), mouse anti-goat horseradish peroxidase (HRP)-conjugated Ab, and rabbit anti-actin HRP-conjugated Ab (I-19) were provided by Santa Cruz Biotechnology (Santa Cruz, CA).

$CD4^+$ T cell isolation

A 10-ml blood sample of each patient and healthy individuals was collected into tubes containing ethylenediaminetetraacetic acid. To isolate $CD4^+$ cells from whole peripheral blood, we employed the positive biomagnetic separation technique using DETACHaBEADs® M-450 CD4, which were coated with anti-CD4 monoclonal antibody, from Dynal Biotech (Lake Success, NY).

Real-time quantitative PCR (RQ-PCR) analysis of ZAP-70, LAT, and SLP-76 transcript levels in $CD4^+$ T cells

Total RNA was isolated according to the method of Chomczyñski and Sacchi [22]. RNA integrity was confirmed by denaturing agarose gel electrophoresis, and the concentration was quantified by measuring the optical density (OD) at 260 nm. RNA samples (1 μg) were treated with DNase I and reverse-transcribed into complementary DNA (cDNA) using oligo-dT primers. RQ-PCR of ZAP-70, LAT, SLP-76, and β-actin cDNA were conducted in a Light Cycler real-time PCR detection system from Roche Diagnostics GmbH (Mannheim, Germany) using SYBR® Green I as detection dye, and target cDNA was quantified using relative quantification method. 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 (Table 2). One RNA sample of each preparation was processed without RT reaction to provide a negative control in subsequent PCR.

Table 2 Oligonucleotide sequences used for RQ-PCR analysis

Primer	Sequence (5′-3′ direction)	ENST number http://www.ensembl.org	Product size (bp)
ZAP-70	GTATCTGAAGCTGAAGGCGG AGGGCTTCGATTTCGTCTCTG	00000264972	150
LAT	CCTACGACAGCACATCCTC GGGTAGGAGGTGACAGGTG	00000323081	109
SLP-76	GGGTGCCGATTCTCAGTAAG CCTCTTCGTGGCTTTCTGTC	00000046794	120
β-actin	GCACCACACCTTCTACAATGAGC GGATAGCACAGCCTGGATAGCAAC	00000158302	166

The quantity of ZAP-70, LAT, and SLP-76 transcripts in each sample was standardized by β-actin transcript levels.

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and Western blotting analysis of ZAP-70, LAT, SLP-76, and DNMT1 protein contents in $CD4^+$ T cells

$CD4^+$ lymphocytes were lysed in lysis buffer, and 20 μg of protein was resuspended in sample buffer and separated on 8% Tris-glycine gel using SDS-PAGE. Gel proteins were transferred to nitrocellulose, which was blocked with 5% milk in Tris-buffered saline/Tween. The immunodetection was performed with gout anti-ZAP-70 Ab (1:500), gout anti-LAT Ab (1:500) or goat anti-SLP-76 Ab (1:500), gout anti-DNMT1 (1:500), and appropriate HRP-conjugated mouse anti-goat Ab. The membranes were reblotted with rabbit anti-actin HRP-conjugated Ab in dilution 1:500 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 ECL kit and Hyperfilm ECL Amersham (Piscataway, NJ). The quantities of ZAP-70, LAT, SLP-76, DNMT1, and β-actin proteins were determined based on band OD in the autoradiogram.

Statistical analysis

The significance of differences between groups was determined using nonparametric Mann–Whitney *U* test. Correlation between SLE patient activities scored on the SLEDAI scale or number of affected organ systems and expression of ZAP-70, LAT, SLP-76, and DNMT1 proteins were assessed by Spearman analysis.

Results

Content of ZAP-70, LAT, and SLP-76 transcripts and proteins in $CD4^+$ T cells from SLE patients and healthy individuals

To compare ZAP-70, LAT, and SLP-76 transcripts number in $CD4^+$ T cells from SLE patients and control individuals, we used RQ-PCR analysis. We did not observe significant statistical differences between $CD4^+$ T cell ZAP-70, LAT, and SLP-76 transcript levels in patients and controls (Fig. 1a - c). We also did not find distinctions in ZAP-70 and SLP-76 protein contents between both investigated groups (Fig. 2a,c, and d). However, the LAT protein level was significantly higher in SLE $CD4^+$ T cells than in controls (P=0.006; Fig. 2b and d).

Correlation between contents of $CD4^+$ T cells ZAP-70, LAT, SLP-76, DNMT1 proteins and disease activity expressed in SLEDAI scale or number of affected organ systems

We found that ZAP-70 protein in SLE $CD4^+$ T cells reciprocally correlated with disease activity expressed in SLEDAI scale (R=−0.623, P=0.002) or number of affected organ systems (R=−0.549, P=0.008; Fig. 3a), respectively. We did not observe these correlations for LAT (R=−0.183, P=0.415; R=−0.214, P=0.340) and SLP-76 (R=−0.141, P=0.531; R=−0.169, P=0.451) proteins in SLE $CD4^+$ T cells (Fig. 3b and c).
Because we were able to detect the DNMT1 protein only in $CD4^+$ T cells in 14 patients and in this group we found reciprocal correlation between DNMT1 protein contents in $CD4^+$ T cells and disease activity expressed in SLEDAI scale (R=−0.779, P=0.001) or number of affected organ systems (R=−0.617, P=0.019), respectively (Fig. 4a and b).

Discussion

To date, Enyedy et al. [23] reported that T cells from SLE patients displayed low level of CD3-ζ protein replaced by FcRIgamma chain in TCR/CD3 complex. Intracellular contents of LAT and SLP-76 proteins and their phosphorylation level also play a key role in distal signal protein activation, regulation of gene expression, and proper function of $CD4^+$ T cells. Cellular content of LAT and SLP-76 proteins and their phosphorylation level is elevated in effector T cells [8, 24]. However, content of these linker/

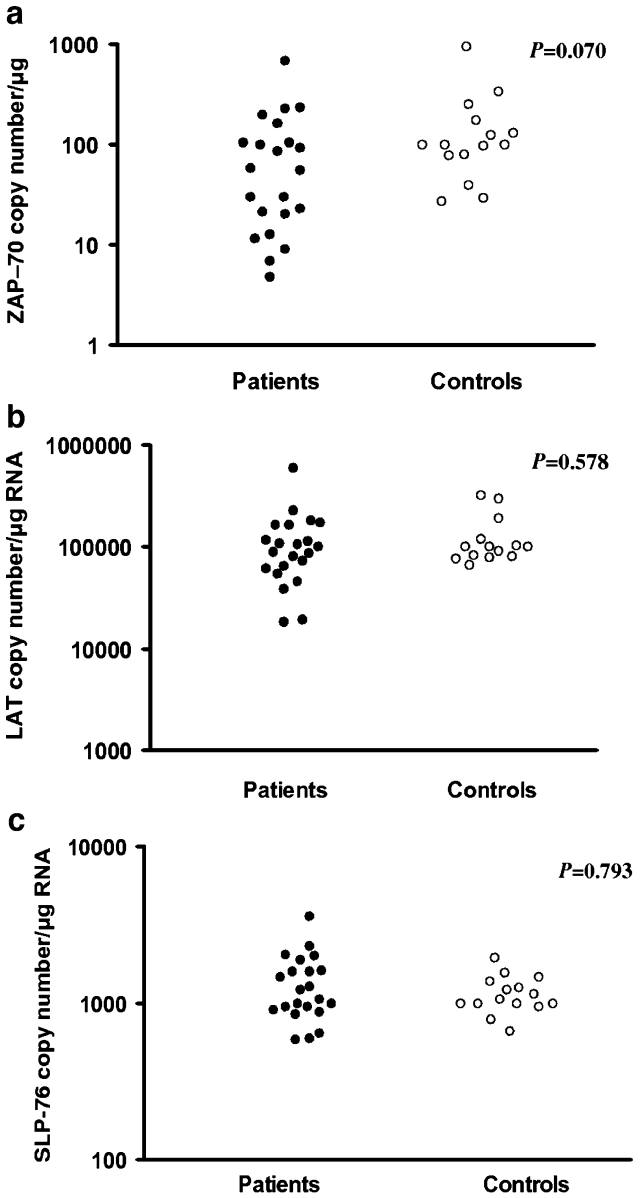

Fig. 1 ZAP-70 (**a**), LAT (**b**), and SLP-76 (**c**) transcripts copy number in $CD4^+$ T cells from SLE patients and controls. The $CD4^+$ T cells that were isolated from peripheral blood by positive biomagnetic separation technique, immediately used for RNA isolation, reverse-transcription, and cDNA, were investigated by RQ-PCR analysis. To normalize the quantity of transcripts in each sample, copy numbers were corrected to the amount of β-actin. The amount of mRNA molecules was expressed in number of transcript copies per microgram of RNA. *Filled circles* and *empty circles* represent transcript level in patients and controls, respectively

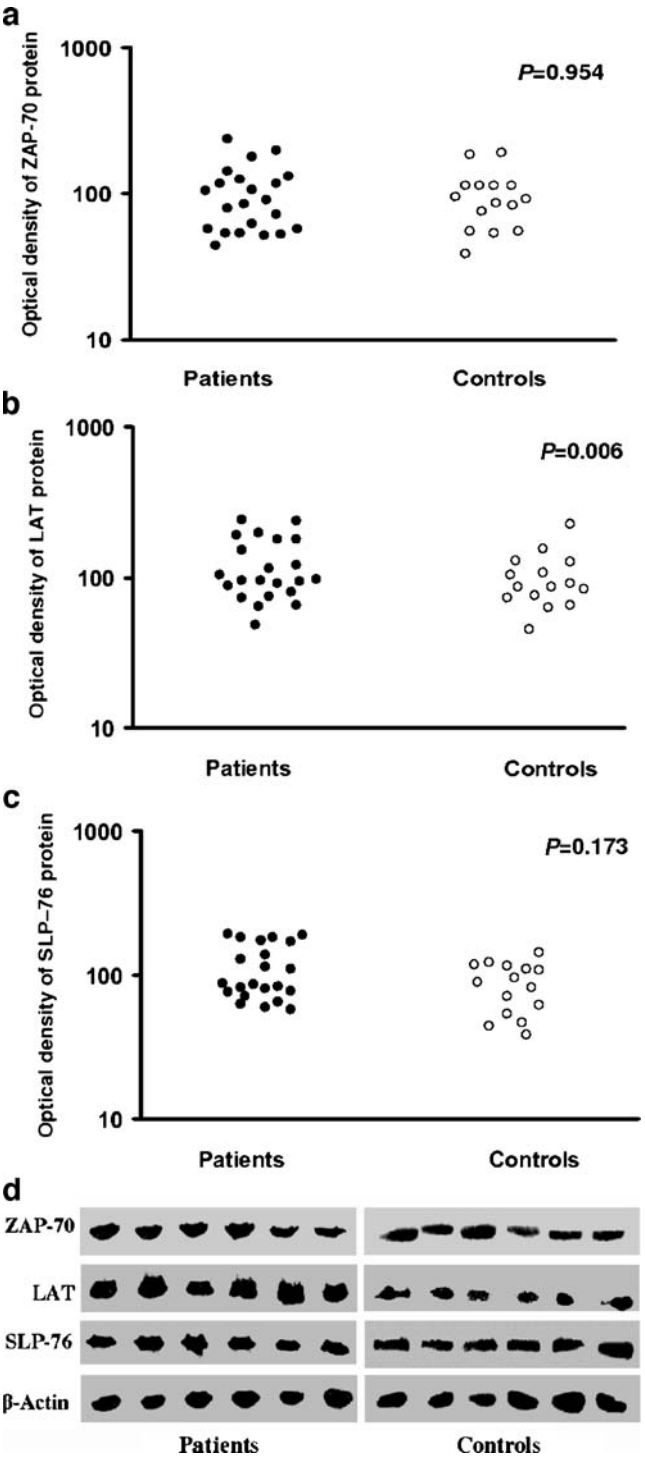

Fig. 2 ZAP-70 (**a**), LAT (**b**), and SLP-76 (**c**) protein contents and representative picture of Western blot analysis (**d**) in $CD4^+$ T cells from SLE patients and controls. The $CD4^+$ T cell proteins were separated using SDS-PAGE, transferred, and the membrane was immunoblotted, respectively, with primary Ab and HRP-conjugated secondary Ab. The membranes were reblotted with anti-β-actin HRP-conjugated Ab to equalize protein loading of the lanes. The quantities of Western blot-detected ZAP-70, LAT, and SLP-76 proteins were determined based on the band OD in the autoradiogram and normalized to β-actin. *Filled circles* and *empty circles* represent $CD4^+$ T cells protein level expressed as band OD in patients and controls, respectively

adaptor proteins has not been investigated in $CD4^+$ T cells from SLE patients.

We compared $CD4^+$ T cells ZAP-70, LAT, and SLP-76 transcript and protein levels between SLE patients and healthy individuals. Despite not finding significant differences in ZAP-70, LAT, and SLP-76 transcript levels (Fig. 1a–c), we observed significant increase in LAT

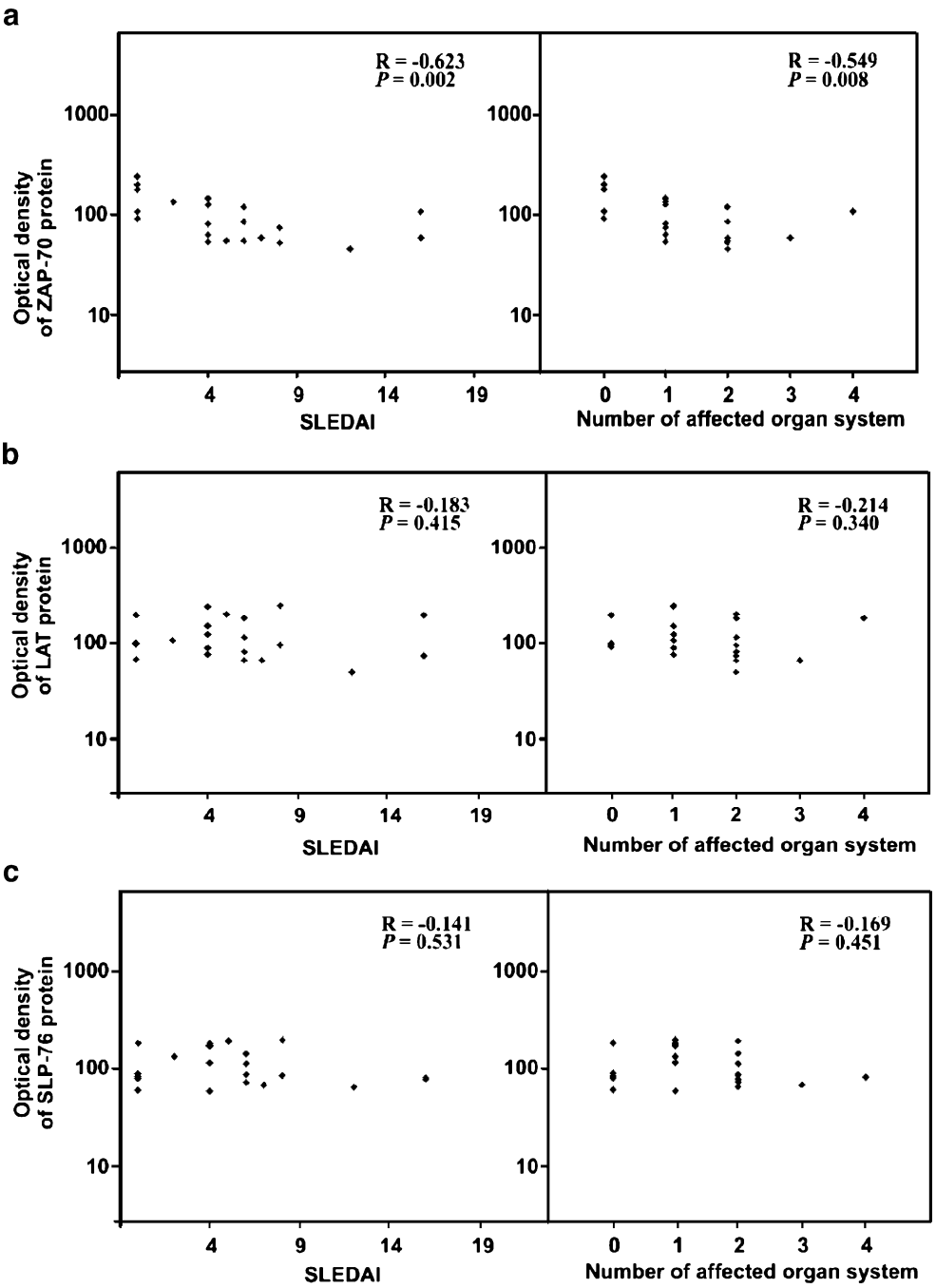

Fig. 3 The correlation between ZAP-70 (**a**), LAT (**b**), and SLP-76 (**c**) protein levels in $CD4^+$ T cells and clinical activity of SLE scored in SLEDAI scale or number of affected organ systems was assessed by Spearman analysis. The $CD4^+$ T cell proteins were separated using SDS-PAGE, transferred, and the membrane was immunoblotted respectively, with primary Ab and HRP-conjugated secondary Ab. The membranes were reblotted with anti-β-actin HRP-conjugated Ab to equalize protein loading of the lanes. The amount of Western blot-detected ZAP-70, LAT, and SLP-76 proteins was determined based on the band OD in the autoradiogram and normalized to β-actin. *R* is Spearman correlation coefficient

protein content in SLE $CD4^+$ T cells compared to controls (Fig. 2b and d). Krishnan et al. [25] did not detect differences between T cell LAT protein level from patients with SLE and controls. However, they investigated all populations of T cells without distinction of $CD4^+$ and $CD8^+$ T cell subsets. On the other hand, in activated $CD4^+$ T cells, LAT level is elevated in comparison to naive T cells [8, 24]. As patients with SLE have higher peripheral blood, $HLA\text{-}DR^+CD4^+$ T cell counts than healthy individuals, this may explain increased contents of LAT protein in SLE $CD4^+$ T cells [26]. As LAT transcript levels in our findings did not significantly differ between $CD4^+$ T cells of patients and controls, this might suggest up-regulation at translational level or decrease in proteolytic degradation of this protein.

To date, the expression of *SLP-76* has not been investigated in $CD4^+$ T cells in SLE patients [8]. Unfortunately, our studies did not find significant differences in $CD4^+$ T cells SLP-76 transcript and protein levels between both analyzed groups (Fig. 2c and d).

Despite not detecting significant ZAP-70 protein differences between the two examined groups (Fig. 2a and d), we

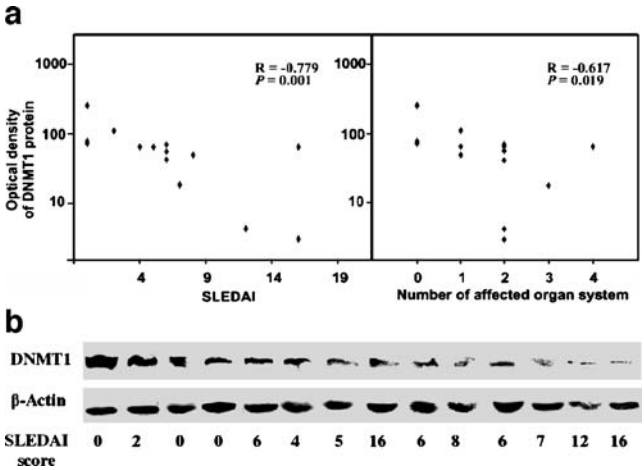

Fig. 4 The Spearman correlation between DNMT1 protein levels and clinical activity of SLE scored in SLEDAI scale or number of affected organ systems (**a**), DNMT1 Western blot analysis (**b**) in $CD4^+$ T cells from SLE patients. The $CD4^+$ T cell proteins were separated using SDS-PAGE, transferred, and the membrane was immunoblotted with goat anti-DNMT1 Ab and mouse anti-goat HRP-conjugated Ab. The membranes were reblotted with anti-actin HRP-conjugated Ab to equalize protein loading of the lanes. We were able to detect the DNMT1 protein in $CD4^+$ T cells from 14 patients only. The amount of Western blot-detected DNMT1 protein was determined based on the band OD in the autoradiogram and normalized to β-actin. The numbers *1–14* correspond to DNMT1 proteins content in $CD4^+$ T cells from 14 patients arrayed with gradual decrease in DNMT1 band OD in the autoradiogram. *R* is Spearman correlation coefficient

found that $CD4^+$ T cell ZAP-70 content correlated with disease activity (Fig. 3a). Krishnan et al. [12] did not observe any change in ZAP-70 protein level during in vitro-activated and 72-h cultured $CD4^+$ T cells [27]. In contrast, Penna et al. [28] detected degradation of ZAP-70 protein by calcium-dependent serine protease-calpain after T cell activation. We suppose that in vivo constant $CD4^+$ T stimulation by self-antigens in patients with SLE may lead to decrease in ZAP-70 protein level, correlating with disease activity. Intracellular reduction in ZAP-70 may resemble decrease in CD3 and CD4 cell surface density as a response to attenuation of permanent stimulation and defense against strong immune response [29, 30]. Lack of statistical differences between $CD4^+$ T cells ZAP-70 protein level between our patient and control groups might be due to small size or low average disease activity of SLE group.

DNMT1 is biosynthesized in S phase of the cell cycle and contributes to methylation of newly formed DNA strand [17, 31]. To date, it has been determined that cellular amounts of this enzyme are significantly reduced in phytohemagglutinin stimulated in vitro SLE T cells vs controls [32, 33]. This fact is consistent with our observation that DNMT1 protein levels in freshly isolated peripheral $CD4^+$ T cells reciprocally correlated with SLE activity or number of affected organ systems (Fig. 4a and b).

Our findings suggest that not only is $CD4^+$ T cell CD3-ζ and FcRIgamma skewed expression associated with SLE disease, but also that $CD4^+$ T cell ZAP-70 and LAT protein content can be also linked with this disease. Our observations were obtained from a relatively small group of SLE patients. Therefore, our results require confirmation in a significantly greater group of patients with SLE, as well as investigation of the same parameters in rheumatoid arthritis or scleroderma patients.

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