Published in final edited form as:

Lupus. 2013 March ; 22(3): 297–306. doi:10.1177/0961203313475691.

Aberrant regulation of the integrin very late antigen-4 in systemic lupus erythematosus

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Abstract

Integrin very late antigen-4 (VLA4) is induced during inflammation and can regulate monocyte migration. It has been implicated in atherogenesis, a significant concern in systemic lupus erythematosus (SLE). The aim of this study was to define VLA4 expression in SLE monocytes. Flow cytometry, reverse transcription polymerase chain reaction, Western blotting, and immunohistochemistry staining with confocal microscopy were used to evaluate VLA4 expression in SLE patients and controls. We found elevated expression of VLA4 in SLE patients with significantly increased VLA4 staining intracellularly compared to control. Exposure of control monocytes to SLE sera or immune complexes led to increased intracellular expression, and immune complexes were capable of driving redistribution of surface VLA4 to the cytoplasm. Therefore, VLA4 was found to be subject to complex regulation with SLE sera driving both RNA expression and redistribution of protein. Stimulation of SLE monocytes with a VLA4 ligand induced significant TNF α expression, confirming a functional effect. This behavior may contribute to increased atherosclerosis and monocyte infiltrates in end organs.

Keywords

Integrin; monocyte; systemic lupus erythematosus; atherosclerosis; immune complex

Introduction

Systemic lupus erythematosus (SLE) is a multiorgan autoimmune disease primarily affecting young women. Premature atherosclerosis and secondary cardiovascular disease are the leading causes of death in SLE.^{1–4} VLA4 has been found to be up-regulated on monocytes from subjects with atherosclerosis and is induced by both inflammatory cytokines and cholesterol.^{5–12} Little is known about VLA4 expression specifically in SLE,^{13,14} but adhesion molecules were found to be globally upregulated in monocytes in our

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Conflict of interest

None declared.

previous studies of RNA expression and histone marks.¹⁵ In addition, other adhesion molecules have been implicated in genome-wide association studies.^{16–18}

Adhesion molecules allow leukocyte adhesion and rolling along endothelial cell surfaces and control migration of leukocytes into inflamed tissues.^{14,19,20} The integrin family consists of $\alpha\beta$ heterodimeric transmembrane glycoproteins and includes cell surface receptors for extracellular matrix components as well as receptors involved in various aspects of leukocyte adhesion.²¹ Integrins are divided into major subfamilies based on the β subunit, including the very late antigen (VLA) family of proteins, of which VLA4 is a member. VLA4 is composed of an $\alpha 4$ (CD49d) and a $\beta 1$ (CD29) subunit and is unique in that it is the only known VLA involved in both cell-cell and cell-matrix adhesion.²² The $\alpha 4$ subunit of VLA4 can be cleaved at its C terminal, with the subunits displaying similar activity as the full protein.^{23,24} The major ligands of VLA4 are vascular cell adhesion molecule-1 (VCAM-1) and fibronectin. VCAM-1 has been studied extensively as a potential marker of disease activity in inflammatory conditions,^{25,26} including SLE nephritis²⁷ and atherosclerosis.²⁸ Upregulation of VLA4 may be mediated by mitogen-activated protein (MAP) kinases in some circumstances.²⁹

Monocytes exhibit broadly abnormal behaviors in SLE, and some data suggest skewing of monocyte subsets.^{30–35} One monocyte subset is defined by intermediate to high CD14 and CD16 (CD14+CD16+) activity and these monocytes have pro-inflammatory characteristics. They have been termed “inflammatory” monocytes. A second subset with little to no CD16 (CD14+CD16–) is considered to be more involved in tissue repair and is sometimes termed “patrolling.”^{36–38} Studies in mice and humans have suggested that monocyte subsets can also be delineated based on expression of the chemokine receptor CX3CR1,³⁹ with increased expression noted on inflammatory monocytes.

We conducted experiments that led to the novel finding of increased cytoplasmic expression of VLA4 in SLE monocytes. Immune complexes could drive intracellular localization of VLA4, while an as yet unidentified component of SLE serum could drive increased expression of VLA4 RNA.

Materials and methods

Patient data

The SLE patients in this study were recruited from a well-known cohort⁴⁰ in which a cumulative SLE database has been constructed for each patient. We specifically recruited subjects with low disease activity and who were not receiving high-level immune suppression or biologic therapy. In addition to nonsteroidal anti-inflammatories, antimalarials, and low-dose prednisone, two patients were on methotrexate and one patient was being weaned from mycophenolate mofetil. Details of the patients recruited for this study are summarized in the Table. Not all patients had all studies performed, and the number studied is given for each analysis in the figure legend. Controls were self-declared as healthy and not on any chronic medications. Patients and controls provided consent as part of an institutional review board (IRB)-approved protocol. SLE serum was pooled from six patients and used as a stimulus for some experiments.

Cells and flow cytometry

Human monocytes with purity >90% were isolated by adherence as previously described.^{41,42} Sera from six patients was collected, pooled, and used for serum-treated cells. Care was taken to ensure that all reagents were free of endotoxin. Controls and SLE samples were handled in parallel with equivalent pre-assay times. Each blood sample was assayed separately. Purified monocytes were used for all experiments except flow cytometry. Pilot studies demonstrated that VLA4 surface expression was somewhat diminished upon culture and handling of cells. Therefore, whole blood samples were used for the flow cytometry studies. Cell surface marker expression was evaluated by standard flow cytometry using a Fluorescence Activated Cell Sorter (FACS LSRII) cytometer (Becton Dickinson, San Jose, CA), FACSDiva software, and FlowJo software. Cells were fixed in 1% paraformaldehyde prior to analysis. Isotype-specific controls were included in each experiment. Monoclonal antibodies for cell surface staining included anti-CD14 (BD Biosciences, San Diego, CA), anti-CD16 (BD Biosciences), anti-CD49d (BD Biosciences), anti-CD29 (BD Biosciences), and anti-CX3CR1 (BioLegend, San Diego, CA).

To stimulate cells with fibronectin, monocytes were incubated on fibronectin-coated wells or uncoated plastic wells and then stimulated with 10 ng/ml of lipopolysaccharide (LPS), 1 µg/ml of immune complexes (ICs) for six hours. A tumor necrosis factor (TNF)-specific enzyme-linked immunosorbent assay (ELISA) was performed on supernatants. The ICs were produced from rabbit anti-bovine serum albumin (BSA): BSA.⁴³ To engage VLA4 in fluid phase, monocytes were incubated with fibronectin at a concentration of 10 µg/ml for one hour.

Western blots

Human monocyte lysates were prepared using lysis buffer (1 × phosphate-buffered saline (PBS)/1% IGEPAL (Sigma-Aldrich, MO, USA)/0.5% sodium deoxycholate/0.1% sodium dodecyl sulfate (SDS))/1mM phenylmethylsulfonyl fluoride (PMSF)/0.2mM sodium orthovanadate/0.5 ug/ml aprotinin). Protein concentration was quantified using the Bradford protein assay (Bio-Rad Laboratories, Hercules, CA) and β actin was used as a loading control. Immunoblotting was performed using anti-CD49d (Santa Cruz Biotechnology, Santa Cruz, CA) at a 1:4000 dilution and anti-CD29 (Santa Cruz Biotechnology) at a 1:2000 dilution as primary antibodies. Secondary antibodies of horseradish peroxidase-conjugated anti-goat and anti-mouse (Santa Cruz Biotechnology) were used at a 1:5000 dilution. Scanned images were quantified using ImageJ 1.43 software (National Institutes of Health, USA).

Surface biotinylation was performed as previously described.^{44–46} Fifty million monocytes were washed with cold PBS twice and rocked at 4° for a half hour. Biotin was added to a final concentration of 1 mg/ml and the cells were rocked for another half hour. The biotin reaction was stopped by adding 5 mM Tris in PBS and the cells were washed extensively in ice-cold PBS. The cells were stripped with glutathione to remove the surface biotin or incubated with 33 ng/ml of ICs for the indicated times. Primaquin was used at 0.3 mM to block recycling back to the surface. Cells were stripped with fresh glutathione twice and then lysed. Streptavidin agarose beads were used to collect biotinylated proteins and equal

amounts of samples were run on a gel. Integrin $\alpha 4$ was detected as described above in a Western blot.

Immunofluorescence

Monocytes were fixed in 4% paraformaldehyde for 10 minutes, blocked with 1% BSA in PBS for one hour, then incubated with primary antibodies (anti-CD49d, anti-CD29) at 1:100 (for CD49d) and 1:50 (for CD29) solutions. The samples were then stained with donkey-anti-goat IgG, 568 Alexa-Fluor and goat-anti-mouse IgG, 647 Alexa-Fluor for one hour in the dark and counter-stained with 4'-6-diamidino-2-phenylindole (DAPI). Confocal images were acquired at 2 μ m optical increments using 488 and 543 lasers. Analysis of fluorescence was performed with Volocity image analysis software (Improvision, MA, USA). BSA-anti-BSA immune complexes were formed as described previously.⁴³ Fluorescent imaging of fibronectin-treated cells was performed on a Zeiss Axio Observer D1 with an X-Cite Series 120 lamp and images were collected using AxioVision.

Real-time polymerase chain reaction (PCR)

Total RNA was isolated using TRI Reagent (Sigma-Aldrich, MO, USA) and RNeasy kit (Qiagen, Hilden, Germany). RNA was reverse transcribed using the Advantage RT-for PCR kit (Clontech Labs, CA, USA). ITGA4 and ITGB1 gene expression was detected by real-time PCR. Relative expression of mRNA transcripts was normalized to the 18 S signal. Commercially available primers were purchased from Applied Biosystems.

Statistics

Statistics were performed using GraphPad Prism 4.0 c software for Mac (GraphPad Software, Inc). Differences between groups were determined using non-parametric tools for paired and unpaired data. Analysis of variance was used when comparing three or more groups. Statistical significance was defined as $p < 0.05$.

Results

There is increased gene and protein expression of integrin $\alpha 4\beta 1$ (VLA4) in SLE monocytes

We first wished to confirm our array studies demonstrating increased ITGA4-ITGB1 RNA expression (corresponding to $\alpha 4\beta 1$) in SLE monocytes compared to control monocytes. We examined RNA expression by evaluating both ITGA4 and ITGB1 cDNA in eight SLE patients and eight controls (Figure 1(a)). We found there was significantly increased ITGA4-ITGB1 mRNA expression in the population with SLE.

Western blot analysis of $\alpha 4$ was then performed to compare levels of protein between SLE monocytes and control monocytes (Figure 1(b)). The $\alpha 4$ subunit can have three bands due to cleavage near its C terminus.³⁸ Since the large subunits have been found to have the equivalent activity level as the total protein, both the 152 and 72kD bands were quantitated (Figure 1(c)). Our data showed that there was significantly increased $\alpha 4$ subunit expression. The $\beta 1$ subunit was also analyzed and showed significant differences between the two populations (data not shown). However, the $\beta 1$ subunit can interact with several different alpha subunits,²¹ so alterations in its expression less precisely predict that of VLA4.

Analysis of monocyte subsets

To gain further insights and independently quantitate VLA4 expression, we performed flow cytometry. One study had suggested increased cell surface expression of VLA4¹³ on monocytes in patients with SLE and vasculitis. To minimize medication-related effects, our study cohort was selected to consist of 20 SLE patients with low disease activity (Table 1) and were age- and gender-matched with 11 control subjects. First, we defined monocyte subsets in our populations. This was conducted by analysis of cell surface markers CD14 and CD16. Primarily, two groups of monocytes were noted, CD14⁺CD16⁺ and CD14⁺CD16⁻ (Figure 2(a)). We found very few CD14^{lo} monocytes and did not quantify them. As can be seen in Figure 2(b), we noted a significantly increased population of CD14⁺CD16⁺ monocytes in the SLE patients. This difference was significant when comparing the frequency of CD14⁺CD16⁺ cells across SLE and control populations (Figure 2(c)). To further examine the characteristics of the inflammatory monocytes, we examined CX3CR1 expression levels. CX3CR1 expression was also significantly increased on CD14⁺CD16⁺ monocytes, as expected, but the expression was comparable between SLE and control monocytes (Figure 3(a)). In examining the monocyte subsets for α 4 expression, we noted significantly increased cell surface α 4 expression on CD14⁺CD16⁺ monocytes compared to CD14⁺CD16⁻ monocytes in controls as well as SLE subjects (Figure 3(b)). This suggested that one explanation for increased VLA4 expression could be a shift in monocyte subsets but the magnitude of the monocyte population shift seemed insufficient to explain the mRNA and protein effects demonstrated in Figure 1.

There is increased intracellular VLA4 in SLE monocytes

To resolve the apparent discrepancy of increased VLA4 RNA and protein in SLE monocytes but not significantly increased cell surface expression, we performed immunofluorescence studies to identify intracellular VLA4 expression. We isolated monocytes from 20 SLE patients and 11 controls and stained for both subunits of VLA4 (Figure 4(a)). Confocal microscopy allowed us to take high-power images at 63 $\times$ magnification with oil submersion, so each cell could be visualized and its intracellular proteins analyzed. We used Volocity image software to quantify immunofluorescence. The intensity of staining per pixel area inside a cell was calculated for 10–15 cells for each sample. The mean fluorescence intensity (MFI) was then calculated. The bar graph (Figure 4(b)) shows the mean of all SLE samples compared to all control samples. Our results showed significantly increased α 4 internal immunofluorescence in SLE monocytes compared to controls, suggesting increased localization of VLA4 in the cytoplasm of SLE monocytes. In the representative cells shown, it may be appreciated that the α 4 and β 1 subunits colocalized well. VLA4 appeared diffusely throughout the cytoplasm but there were also discrete focal areas, suggesting some of the protein may reside in cytoplasmic vesicular compartments. Experiments using markers specific for early endosomes did not show colocalization with VLA4, suggesting these compartments are not early endosomes (data not shown).

The presence of ICs alters VLA4 expression in monocytes

In an effort to determine if external factors in the SLE milieu affected VLA4 expression in monocytes, we incubated control monocytes in SLE sera (Figure 5(a)). The results showed

increased internal $\alpha 4$ immunofluorescence in SLE sera-exposed monocytes using Volocity to quantitate the pixels ($p < 0.05$) (Figure 5(b)). To further define variables governing intracellular localization, we treated control monocytes with ICs and noted increased intracellular staining (Figure 5(a) and 5(b)) when compared to control monocytes. We also noted increased RNA expression when control monocytes were incubated with SLE sera (Figure 5(c)). To further explore the effect of ICs on internal VLA4 expression, control monocytes were treated with ICs, and VLA4 internalization was measured by Western blot after surface biotinylation. IC-treated monocytes showed a decrease in cell surface $\alpha 4$ expression and an increase in intracellular $\alpha 4$ expression when compared to control monocytes (Figure 6). Therefore, soluble constituents within SLE sera drive both the increased transcription and internalization.

Stimulation through VLA4

The cytoplasmic VLA4 could be sequestered and non-functional or could be available for signaling. To examine this, we incubated control and SLE monocytes on conventional or fibronectin-coated wells. Fibronectin is a ligand for VLA4, and incubation with fibronectin alone induced far more TNF α compared to plastic (Figure 7). LPS induction of TNF α on fibronectin appeared to induce no increase over and above that seen with fibronectin, suggesting the effect is powerful. We next incubated monocytes with fluid phase fibronectin and examined cells by fluorescent microscopy for VLA4 internalization. Internalization was not as marked as with ICs although we did observe some aggregates, similar to what was seen after IC treatment (Figure 7).

Discussion

Our work shows that there is a significant difference in monocyte subsets between SLE patients and controls, with SLE patients having more CD14+CD16+ inflammatory monocytes, despite the fact that the patients had low disease activity. In a recent paper by Merino et al., it was noted that the CD14+CD16+ monocyte population has both proinflammatory and proatherosclerotic activity.³⁷ Our work shows that this population also has increased cell surface VLA4 expression, suggesting this monocyte population could have altered migratory abilities. Our data, coupled with studies demonstrating increased expression of VCAM1 in SLE, suggest an overall increased adhesive network in SLE.^{47–49} This could contribute not only to atherosclerosis but also to monocyte infiltration into renal parenchyma and vasculitis.

Further exploration of VLA4 in SLE monocytes noted dramatically increased intracellular VLA4 expression as determined by immunofluorescence. To determine the etiology of this, we first attempted to mimic the effect using SLE serum, noting increased intracellular VLA4 expression in SLE sera-exposed control monocytes and increased ITGA4 mRNA expression from these cells. This suggests that exposure to SLE sera increased VLA4 protein synthesis. Our confocal experiments showed a trend toward increased intracellular VLA4 expression after IC stimulation, and the biotinylation experiment demonstrated that ICs could drive a redistribution of VLA4 to an intracellular pool. The function of this intracellular pool is completely unknown, although stimulation with fibronectin on a solid substrate led to much

higher TNF α production by SLE monocytes than control monocytes, suggesting that the intracellular pool can be recruited for functional responses.

Our demonstration of increased VLA4 expression in SLE was driven by previous array studies of lupus monocytes.^{15,50} The studies reported here confirm the importance of adhesion molecules in the pathogenesis of SLE. Genome-wide association studies also point to susceptibility that maps to adhesion molecules.^{51,52} Surprisingly, the effect was robust in this clinically quiescent study population. This effect would be predicted to enhance atherogenic activities and cardiovascular disease in the SLE population, even with apparently clinically quiet disease. Taken together, these data provide a context for considering novel therapeutics targeted to the VLA4 pathway to treat and prevent atherosclerosis in SLE.

Acknowledgments

The authors gratefully acknowledge the support of the patients and the staff who care for them. The authors would like to thank Drs. Edward Behrens and Terri Finkel for helpful advice.

Funding

This work was supported in part by 2R01 ES017627 and T32-AR007442-21A1.

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Figure 1. Analysis of VLA4 expression in control and SLE monocytes

(a) VLA4 cDNA was measured as the $\alpha 4$ (CD49d) subunit (ITGA4) and the $\beta 1$ (CD29) subunit (ITGB1) by qRT-PCR. Expression in SLE monocytes is increased (n =five to eight patients, $p<0.05$). Stimulation with lipopolysaccharide (LPS) did not significantly alter expression. Error bars represent standard deviation from the mean. (b) Representative Western blots of VLA4 $\alpha 4$ for 11 control patients and 11 SLE patients are shown. Monocyte lysates were analyzed for the $\alpha 4$ subunit with anti-CD49d, which can detect the mature protein (152 kD) or the cleaved C' terminal (72 kD). β actin served as a loading control. Red blood cell lysates were used as negative control on a different Western blot to ensure specificity of the antibody. (c) VLA4 $\alpha 4$ protein expression was quantitated with ImageJ. There was increased expression in SLE monocyte lysates compared to controls ($n=14$ SLE and 13 control samples, $p=0.03$). Columns represent the mean ratio of $\alpha 4$ signal to actin. Error bars represent standard deviation from the mean. VLA4: integrin very late antigen-4; SLE: systemic lupus erythematosus; qRT-PCR: quantitative reverse-transcription polymerase chain reaction.

Figure 2. Analysis of monocyte subsets

(a) Representative flow-cytometry analyses of monocyte subsets assessed using CD16-FITC/CD14-PECy7 with control monocytes and (b) SLE monocytes are shown. Populations were initially gated SSC-height/CD14-PECy7 and backgated to confirm physical parameters. (c) A box-plot demonstrating the percentage of CD14⁺CD16⁺ monocyte subset in SLE patients (*light gray box*) and healthy controls (*dark gray box*). The percentage of CD14⁺CD16⁺ cells is increased in SLE ($n=11$ control, 20 SLE patients; $p<0.05$). Bars are standard deviation from the mean. SSC: Side Scatter; FITC: fluorescein isothiocyanate; SLE: systemic lupus erythematosus.

Figure 3. Expression of VLA4 and CX3CR1 on monocyte subsets

(a) Expression of CX3CR1 is increased on CD14+CD16+ monocytes in control and SLE populations compared to CD14+CD16- monocytes ($n=11$ control, 20 SLE patients). The difference between CXCR1 expression on CD16+ and CD16- negative subsets is significant for SLE and control populations. Asterisks indicate specific comparisons. (b) VLA4 $\alpha 4$, measured by anti-CD49d, has increased expression on CD14+CD16+ monocytes compared to CD14+CD16- monocytes in control and SLE populations ($n=11$ control, 20 SLE patients). The difference between VLA4 $\alpha 4$ expression on CD16+ and CD16- negative subsets is significant for both SLE and control populations. Asterisks indicate specific comparisons. *, ** $p<0.05$. Error bars are standard deviation from the mean. VLA4: integrin very late antigen-4; SLE: systemic lupus erythematosus; MFI: mean fluorescence intensity.

Figure 4. Intracellular expression of VLA4 in monocytes

(a) Expression of $\alpha 4$ (CD49d, red) and $\beta 1$ (CD29, green) in SLE monocytes and control monocytes was defined by confocal analysis. The representative confocal slices were taken from the middle of the cell. BF is the brightfield microscopy image used to outline the cell for quantitation. Increased internal $\alpha 4$ and $\beta 1$ immunofluorescence is seen in SLE monocytes compared to control. (b) Quantitation of intracellular VLA4 in control monocytes compared to SLE monocytes is shown; 10–15 cells were analyzed per sample using intensity finding Volocity software analysis ($n=11$ control, 20 SLE patients; $p<0.05$). Error bars are standard deviation from the mean. VLA4: integrin very late antigen-4; SLE: systemic lupus erythematosus; MFI: mean fluorescence intensity.

Figure 5. Intracellular expression of VLA4 after exposure to stimuli

(a) Representative pictures showing expression of $\alpha 4$ (CD49d, red) and $\beta 1$ (CD29, green) in healthy monocytes after incubation with RPMI + 25% SLE sera, RPMI + 25% healthy human (control) sera, or RPMI media only. One group of healthy monocytes was stimulated with immune complexes (IC) for six hours. Increased internal VLA4 immunofluorescence is seen in SLE sera-exposed monocytes (n =three experiments). (b) Intracellular $\alpha 4$ expression was quantitated. There was significantly increased expression after stimulation with SLE sera compared to control sera or media (n =three separate experiments; *, #; $p<0.05$); 10–15 cells were analyzed per condition per experiment using Volocity software. Error bars are standard deviation from the mean. (c) VLA4 cDNA was measured as the CD49d subunit (ITGA4) and the CD29 subunit (ITGB1) by qRT-PCR. Control monocytes were incubated in RPMI+25% SLE sera, RPMI+25% healthy human (control) sera, or RPMI media only. SLE sera was a cocktail from several patients with similar disease activity. Expression in SLE sera-treated monocytes is increased compared to monocytes treated with control sera or RPMI media alone (n =three separate experiments, *, #; $p<0.05$). Error bars represent standard deviation from the mean. VLA4: integrin very late antigen-4; RPMI: Roswell Park Memorial Institute; SLE: systemic lupus erythematosus; MFI: mean fluorescence intensity; qRT-PCR: quantitative reverse-transcription polymerase chain reaction; IC: Immune Complexes.

Figure 6. Surface biotinylation of VLA4

Cell surface proteins on control monocytes were biotinylated and stimulated with immune complexes. At various time points, cell surface biotin was stripped. Biotinylated proteins recovered represent intracellular proteins. In this experiment, representative of five separate experiments, immune complexes drive internalization of VLA4 $\alpha 4$. VLA4: integrin very late antigen-4.

Figure 7. VLA4 engagement

(a) SLE monocytes or control monocytes were incubated on fibronectin-treated wells or uncoated wells ($n=$ four each). Some wells were treated with 10 ng/ml of LPS. Triplicate wells of each treatment were harvested after six hours for three separate experiments and run on a human TNF α ELISA plate with standards. Fibronectin alone induced more TNF α in the SLE monocytes than the control monocytes ($p=0.02$). There was little effect on LPS induction. (b) To better understand the effect of fibronectin, control cells were treated with fibronectin in solution and then stained for VLA4 internalization. Fibronectin did induce VLA4 internalization although to a more limited extent than that seen with immune complexes ($n=$ two). VLA4: integrin very late antigen-4; SLE: systemic lupus erythematosus; LPS: lipopolysaccharide; TNF: tumor necrosis factor; ELISA: enzyme-linked immunosorbent assay.

Table 1Demographic characteristics of the study population ($n=40$)

Characteristic	Average
Age (year): mean (range)	44 (24–74)
% Female	90
% Race (Caucasian/Black)	70/30
Mean SLEDAI (range)	2 (0–7)
Mean ESR (mm/h): range	31: 2–87
Mean C3 (g/l): range	106: 69–176
Mean C4 (g/l): range	19: 2–41
Mean prednisone dose (mg/d): range	4: 0–30
Anti-dsDNA	0

SLEDAI: Systemic lupus erythematosus disease activity index; ESR: erythrocyte sedimentation rate; anti-dsDNA: anti-double-stranded DNA.