Original Article

Association of Disease Activity with Programmed Cell Death 1 and Its Ligand Programmed Cell Death Ligand 1 Expressions in Lupus Patients

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Abstract

Background: Programmed cell death 1(PD-1)/programmed cell death ligand 1 (PD-L1) pathway is an immune checkpoint implicated in immune tolerance and involved in the pathogenesis of several autoimmune diseases. Systemic lupus erythematosus (SLE) is an autoimmune disease with multiple immune dysregulation. This study aimed to determine PD-1 and PD-L1 expressed levels on both CD3 T and CD19 B lymphocytes in SLE patients compared to healthy donors and their associations with the clinical data and disease activity of those patients.

Patients and Methods: A total of 25 healthy donors and 80 SLE patients were involved in the study. PD-1 and PD-L1 expressed levels on each of CD3 T and CD19 B lymphocytes were determined in the peripheral blood (PB) using flow cytometry.

Results: The expressed levels of PD-1 and PD-L1 on both CD3 T and CD19 B lymphocytes were significantly higher in PB of SLE group than that of controls ($P = 0.01$, $P = 0.001$, $P = 0.009$, and $P = 0.001$). Significant positive associations were found between PD-1 and PD-L1 expressions on both CD3 T and CD19 B lymphocytes with disease activity in SLE group ($P < 0.05$).

Conclusion: PD-1 and its ligand PD-L1 could have a role as regulators for immune activation in patients with SLE.

Key Words: *B lymphocytes, programmed cell death 1, programmed cell death ligand 1, systemic lupus erythematosus, T lymphocytes*

Introduction

The immune system is characterized by having many co-stimulatory and co-inhibitory pathways that regulate the immune response. High co-stimulation and/or inadequate co-inhibition may result in a defect of self-tolerance and thus to autoimmunity.[1]

Programmed cell death 1 (PD-1) is a member of CD28 superfamily. It is an immune checkpoint molecule and expressed on the surface of activated human lymphocytes, natural killer cells, activated monocytes, and myeloid cells.[2] Binding PD-1 to its ligand programmed cell death ligand 1 (PD-L1) elicits an inhibitory signal toward the activated immune lymphocytes, expressing PD-1 reducing their activation and proliferation; downregulates the immune responses; maintains peripheral tolerance; and protects tissues from inflammatory or autoimmune attack.[3-6] The PD-1 pathway regulates T-lymphocyte tolerance in various ways. It reduces activation and expansion of self-reactive T lymphocytes and blocks the function of self-reactive T-cell effector and organ damage. PD-1 binding to its ligand can decrease expansion and differentiation of naive self-reactive T lymphocytes. Expression of PD-1 ligands on tolerogenic dendritic cells provides a means for controlling between T-lymphocyte activation and tolerance.[7,8]

Interestingly, several evidences reported mainly from experimental models demonstrated the importance of the PD-1/PD-L1 axis in modulating autoimmunity.[9-11] PD-1/PD-L1 pathway role has been evaluated in different autoimmune diseases such as Type 1 diabetes,[12] systemic lupus erythematosus (SLE),[13] and rheumatoid

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arthritis (RA).[14] In addition, genetic variations of PD-1 gene have been documented to associate with the risk of autoimmune diseases, such as SLE, RA, and Graves' disease.[15-17] The immune regulation reported by PD-1 pathway in experimental models showed that its genetic lack in mice (pdcd1-/-) led to the emergence of arthritis, dilated cardiomyopathy, or lupus-like autoimmune disease.[18-20]

SLE is a heterogeneous autoimmune disease characterized by chronic activation of immune system and production of auto-antibodies against self-antigens such as nuclear components.[21-23]

Our work aims to determine PD-1 and PD-L1 expressions on both CD3 T and CD19 B lymphocytes in SLE patients' peripheral blood (PB) versus controls. Also, we investigate the associations of PD-1 and PD-L1 expressions with the clinical and laboratory data and disease activity of patients.

Patients and Methods

Ethics

This study was approved by the ethics committee of National Research Centre, Cairo, Egypt, with an approval number 20167, and has therefore been performed in accordance with the ethical standards laid down in the 1964 Declaration of Helsinki and its later amendments. All samples were obtained with the written informed consents of the subjects.

Study subjects

This case–control study included 25 healthy subjects and 80 SLE patients recruited from the Rheumatology and Rehabilitation outpatient clinic, Kasr Al Ainy Hospital, Cairo University, from October 2019 to February 2020. All patients fulfilled the American College of Rheumatology criteria for SLE.[24] Demographic and cumulative clinical manifestations were recorded, and disease activity was assessed through the Safety of Estrogens in Lupus Erythematosus National Assessment-SLE Disease Activity Index (SELENA-SLEDAI).[25] Flare using SELENA-SLEDAI is defined as follows: No flare present ≤3, mild or moderate flare 3–12, and severe flare >12.

Flow cytometry analysis

The PB was collected from patients and healthy controls in tubes containing EDTA. 50 μL of EDTA-treated PB was incubated for 30 min at 4°C in the dark with fluorochrome-labeled monoclonal antibodies (mAbs): fluorescein isothiocyanate-conjugated CD3, PerCP-conjugated CD19, phycoerythrin (PE)-conjugated CD279 (anti-PD-1 mAB), and allophycocyanin-conjugated CD274 (anti-PD-L1 mAB) (Becton Dickinson, USA). The red blood cells were lysed using BD FACS Lysing Solution (Becton Dickinson, USA). The stained cells were then washed and resuspended in phosphate-buffered saline. Approximately 30,000 stained cells in each sample were analyzed with a BD FACSCanto 10 flow cytometer (BD Biosciences). The lymphocytes were gated by setting the appropriate forward scatter/side scatter axes. Data were acquired, and data analysis was performed by the FACS DIVA software program.

Statistical analysis

Data were statistically analyzed using SPSS version 19.0 software (SPSS Inc., Chicago, IL, USA). Nonparametric Mann–Whitney U-test was used to compare the expression of PD-1 and PD-L1 on T and B cells between groups. The correlation of PD-1 and PD-L1 expressions with disease activity and proteinuria of patients was performed using Spearman's correlation analysis. Data were presented as the mean ± standard deviation or median (interquartile range). A $P < 0.05$ was considered statistically significant.

Results

Clinical and laboratory characteristics of systemic lupus erythematosus patients

Eighty SLE patients were enrolled in the study, with a mean age at the time of sampling being 30 ± 7.4 years, and the median disease duration (interquartile range) was 6 (9) years. Seventy-four (92.5%) patients were females. Cumulative clinical manifestations, laboratory findings, and medications of patients at the time of sampling are summarized in Table 1.

Percentages and mean fluorescence intensity of programmed cell death 1 CD3 T lymphocytes and programmed cell death ligand 1 CD3 T lymphocytes

Our findings demonstrated that PD-1 and its ligand PD-L1 expressions on CD3 T lymphocytes were significantly elevated in patients compared to controls [Figure 1]. In PB of SLE patients, a percentage of PD-1 CD3 T lymphocytes was upregulated ($P = 0.171$), while the mean fluorescence intensity (MFI) of them showed a significant increase ($P = 0.01$) compared to controls. Moreover, statistical analysis of percentage and MFI of PD-L1 CD3 T lymphocytes showed a significant upregulation in lupus patients versus normal donors ($P = 0.002$ and $P = 0.001$, respectively).

Percentages and mean fluorescence intensity of programmed cell death 1 CD19 B lymphocytes and programmed cell death ligand 1 CD19 B lymphocytes

Our results showed that CD19 B lymphocytes had a high percentage in PB of SLE patients in comparison to controls ($P = 0.616$). Significantly elevated expression levels of PD-1 and PD-L1 on CD19 B lymphocytes were detected in SLE group relative to controls [Figure 2]. A percentage and MFI of PD-1 CD19 B lymphocytes

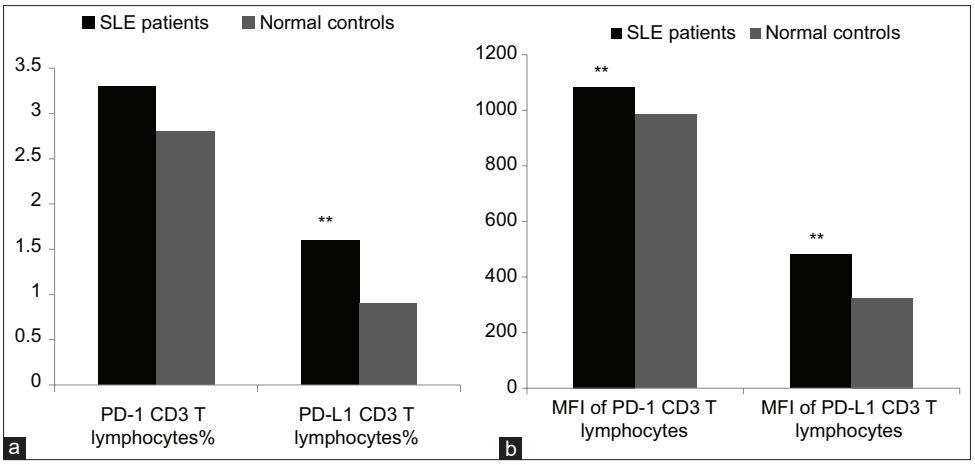

Figure 1: Programmed cell death 1 and programmed cell death ligand 1 expressions on CD3 T lymphocytes in peripheral blood of systemic lupus erythematosus patients compared to controls (data were expressed as median). (a) Percentages of programmed cell death 1 CD3 T lymphocytes and programmed cell death ligand 1 CD3 T lymphocytes. (b) The mean fluorescence intensity of programmed cell death 1 CD3 T lymphocytes and programmed cell death ligand 1 CD3 T lymphocytes. **: Statistically significant at $P < 0.05$ versus healthy donors, by Mann–Whitney U-test

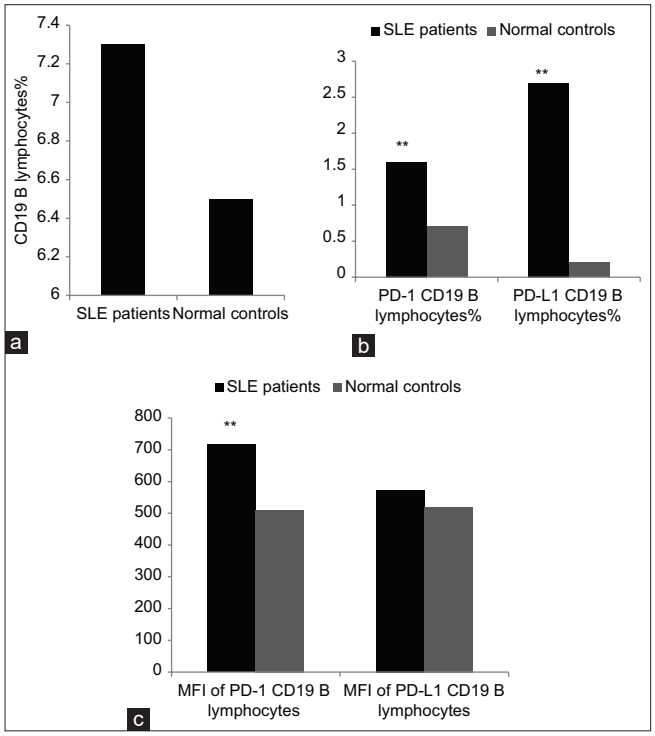

Figure 2: Programmed cell death 1 and programmed cell death ligand 1 expressions on CD19 B lymphocytes in peripheral blood of systemic lupus erythematosus group compared to controls (data were expressed as median). (a) Percentages of CD19 B lymphocytes. (b) Percentages of programmed cell death 1 CD19 B lymphocytes and programmed cell death ligand 1 CD19 B lymphocytes. (c) The mean fluorescence intensity of programmed cell death 1 CD19 B lymphocytes and programmed cell death ligand 1 CD19 B lymphocytes. **: Statistically significant at $P < 0.05$ versus controls, by Mann–Whitney U-test

in PB of SLE patients were significantly upregulated in comparison to controls ($P = 0.009$ and $P = 0.016$, respectively). On the other hand, a percentage of PD-L1 CD19 B lymphocytes was significantly elevated ($P = 0.001$), while MFI of them was slightly increased ($P = 0.528$) compared to controls.

Table 1: Clinical manifestations, laboratory findings, and medications of systemic lupus erythematosus patients

	n (%)
Clinical manifestations	
Nephritis	66 (82.5)
Arthritis	64 (80)
Antiphospholipid syndrome	13 (16.25)
Cutaneous	64 (80)
Serositis	28 (35)
SLEDAI, median (IQR)	4 (11)
Laboratory findings	
ANA	80 (100)
Anti-ds DNA	66 (82.5)
Hypocomplementemia	34 (42.5)
Leucopenia	16 (20)
Proteinuria (g/day), median (IQR)	0.4 (2.5)
Medications	
Steroids	80 (100)
Hydroxychloroquine	80 (100)
Cyclophosphamide	10 (12.5)
Mycophenolate mofetil	28 (35)
Azathioprine	32 (40)

SLEDAI: Systemic lupus erythematosus disease activity index, IQR: Interquartile range, ANA: Antinuclear antibodies, Anti-ds DNA: Anti-double stranded DNA

Association of programmed cell death 1/ programmed cell death ligand 1 expressions with the clinical and laboratory manifestations and disease activity of lupus patients

Our study showed elevation of PD-1 and PD-L1 expressed on CD3 T and CD19 B lymphocytes in patients with lupus nephritis compared to those without nephritis [Table 2]. There was a significant difference in the percentages of PD-1 CD3 T lymphocytes between patients based on grades of flare, and there was an increase in PD-1 and

PD-L1 expression on T and B lymphocytes in parallel with the increase of flare grades [Table 3]. Furthermore, significant positive associations were found between PD-1 and PD-L1 expressions on both CD3 T lymphocytes and CD19 B lymphocytes with both of SLEDAI and proteinuria (24-h urinary protein) in lupus patients [$P < 0.05$, Figures 3 and 4]. Interestingly, percentages of PD-1 CD3 T lymphocytes were positively associated with percentages of PD-1 CD19 B lymphocytes ($r = 0.396$, $P = 0.015$) and also MFI of PD-1 CD3 T lymphocytes was positively associated with MFI of PD-1 CD19 B lymphocytes ($r = 0.402$, $P = 0.014$) in a significant manner.

Discussion

The immune regulatory pathways of immune checkpoints are important in maintaining the homeostasis and tolerance of the immune system. The PD-1/PD-L1 pathway is one of the critically examined immune checkpoints in autoimmune diseases.[14,26,27] It is activated during the activation of immune cells and involved in their proliferation and differentiation,[28] and plays vital roles in reducing the immune response, maintaining tolerance to self-antigens by diminishing activation of T lymphocytes, enhancing apoptosis of stimulated effector T lymphocytes, and lowering regulatory T lymphocyte apoptosis.[29]

Our findings demonstrated that there was a significant elevation in the expressed levels of PD-1 and its ligand PD-L1 on CD3 T lymphocytes in patients compared to controls. In PB of SLE patients, percentages of PD-1 CD3 T lymphocytes were upregulated, while MFI of them was significantly increased compared to controls. Moreover, a percentage and MFI of PD-L1 CD3 T lymphocytes were significantly higher in PB of lupus patients than that of controls. This is in agreement with Liu *et al*. and Stefanski *et al*., who found significantly increased percentages of PD-1-expressing CD3 T lymphocytes with comparable MFI of PD-1 and PD-L1 expressions in PBMCs of SLE group compared with normal donors.[26,30] In another study by Jiao *et al*., PD-1 was highly expressed on T lymphocytes in PB of SLE group compared to controls. They also found higher levels of PD-1 gene expression in SLE group than that of controls.[17]

In this study, percentages of CD19 B lymphocytes were elevated in PB of SLE group compared to controls. Significantly elevated expressed levels of PD-1 and PD-L1 on CD19 B lymphocytes were detected in lupus patients relative to controls. A percentage and MFI of PD-1 CD19 B lymphocytes in PB of SLE group were significantly upregulated in comparison to controls. On the other hand, a percentage of PD-L1 CD19 B lymphocytes was significantly elevated, while MFI of them was slightly

Table 2: Comparison of programmed cell death 1 and programmed cell death ligand 1 expressions on T and B lymphocytes between lupus patients based on the presence or absence of nephritis

Parameter	Nephritis	Percentage**	*P*	MFI**	*P*
PD-1 CD3 T lymphocytes	Presence	5.8 (3.3-15.7)	0.076	1379 (1009-2015)	0.028*
	Absence	3.1 (0.7-7.4)		928 (884-1080)	
PD-L1 CD3 T lymphocytes	Presence	2.7 (1.4-5.6)	0.093	521 (517-595)	0.117
	Absence	1.1 (0.6-4.8)		460 (376-735)	
PD-1 CD19 B lymphocytes	Presence	2.1 (0.4-7.3)	0.754	852 (669-3349)	0.076
	Absence	1.2 (0.3-3.3)		360 (337-1649)	
PD-L1 CD19 B lymphocytes	Presence	8.9 (2.8-9.7)	0.047*	485 (392-584)	0.327
	Absence	1.3 (0.4-9.5)		394 (380-631)	

*Significant at $P<0.05$ (by Mann-Whitney U test), **Results were expressed as median (range). MFI: Mean fluorescence intensity, PD-1: Programmed cell death 1, PD-L1: Programmed cell death ligand 1

Table 3: Comparison of programmed cell death 1 and programmed cell death ligand 1 expressions on T and B lymphocytes between patients based on grades of flare

Parameter	Grade 1*	Grade 2*	Grade 3*
PD-1 CD3 T lymphocytes%	1.3[b, c] (0.7-2.3)	2.9[a, c] (1.8-5)	11[a, b] (9.4-15.7)
MFI of PD-1 CD3 T lymphocytes	928 (884-957)	1115 (921-1379)	1921 (1820-2015)
PD-L1 CD3 T lymphocytes%	1.24 (0.6-2.6)	1.8 (1.1-5.6)	2.7 (2-3.8)
MFI of PD-L1 CD3 T lymphocytes	519 (376-574)	489 (402-521)	606 (450-735)
PD-1 CD19 B lymphocytes%	1.6 (0.3-3.3)	1.9 (0.4-4.2)	5.6 (4.7-7.3)
MFI of PD-1 CD19 B lymphocytes	513 (342-910)	796 (337-1373)	2064 (779-3349)
PD-L1 CD19 B lymphocytes%	1.2 (0.4-4.9)	2.5 (1.3-9.7)	6.2 (5-7.4)
MFI of PD-L1 CD19 B lymphocytes	412 (390-586)	438 (380-519)	495 (390-631)

*Results were expressed as median (range), [a]Significant at $P<0.05$ versus Grade 1, [b]Significant at $P<0.05$ versus Grade 2, [c]Significant at $P<0.05$ versus Grade 3. MFI: Mean fluorescence intensity, PD-1: Programmed cell death 1, PD-L1: Programmed cell death ligand 1

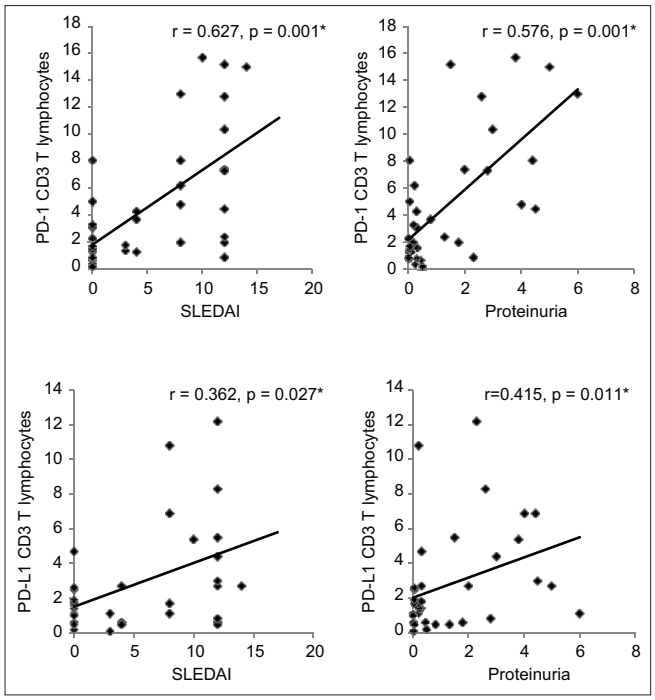

Figure 3: Significant positive correlations between programmed cell death 1 CD3 T lymphocytes and programmed cell death ligand 1 CD3 T lymphocytes with SLEDAI and proteinuria (24 h urinary protein). *: Spearman's correlation analysis

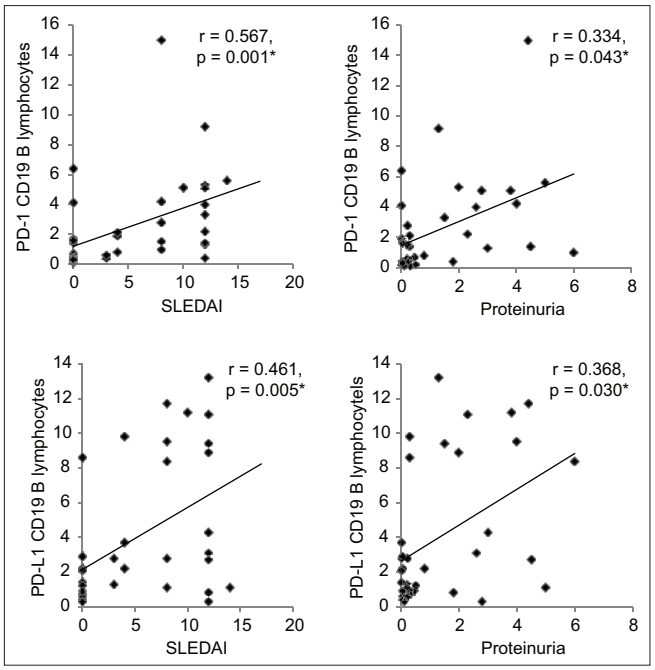

Figure 4: Significant positive correlations between programmed cell death 1 CD19 B lymphocytes and programmed cell death ligand 1 CD19 B lymphocytes with SLEDAI and proteinuria (24 h urinary protein). *: Spearman's correlation analysis

increased compared to controls. This is consistent with several studies that showed a significant upregulation of CD19 B lymphocytes and CD19 B lymphocytes expressing PD-1 and PD-L1 in SLE patients compared to controls.[28,30]

Our results showed an elevation of PD-1 and PD-L1 expressed on CD3 T and CD19 B lymphocytes in patients with lupus nephritis compared to those without nephritis. There was a significant difference in the percentages of PD-1 CD3 T lymphocytes between patients based on grades of flare, and there was an increase in PD-1 and PD-L1 expression on T and B lymphocytes in parallel with the increase of flare grades. Furthermore, significant positive correlations were found between PD-1 and PD-L1 expressions on both CD3 T lymphocytes and CD19 B lymphocytes with both of SLEDAI and proteinuria in lupus patients. Interestingly, percentages of PD-1 CD3 T lymphocytes were positively associated with the percentages of PD-1 CD19 B lymphocytes and also MFI of PD-1 CD3 T lymphocyte was positively associated with MFI of PD-1 CD19 B lymphocytes in a significant manner. This matches with the results of Jia *et al.* who demonstrated elevated numbers of CD19 B-lymphocytes and PD-L1-expressing CD19 B-lymphocytes in SLE group with anti-dsDNA (+) versus those patients with anti-dsDNA (-).[28] Moreover, it has been reported that upregulated PD-1 expression on T lymphocytes and expression levels of PD-1 gene in PBMCs of SLE patient were significantly correlated with SLEDAI scores.[17] Furthermore, Stefanski *et al*. observed a positive correlation between T lymphocytes and B lymphocytes expressing PD-1.[26]

These findings support the hypothesis that PD-1 and PD-L1 disruption contributes to the pathogenesis of lupus. PD-1 gene defects have been reported to increase the susceptibility to autoimmune diseases, such as RA, Graves' disease, and SLE.[15-17] Animal studies have indicated that mice lacking PD-1 gene developed glomerulonephritis, arthritis, or lupus-like disease.[18-20] In recent years, exhausted immune cell-based cancer therapy has been developed such as PD-1 pathway blockers.[31] Immune-related side effects such as autoimmune symptoms have been associated with these PD-1 inhibitors.[32]

Conclusion

PD-1/PD-L1 expressions on CD3 T lymphocytes and CD19 B lymphocytes were significantly upregulated in SLE group compared to normal controls and they were positively associated with the disease activity of patients. These findings strengthen the hypothesis that PD-1 and its ligand PD-L1 could have a role as regulators for immune activation in patients with SLE. This could possibly provide new perspectives for future treatment strategies.

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Nil.

Conflicts of interest

There are no conflicts of interest.

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