A Study of Interleukin-2 Production and Nck Adaptor Molecule Gene Expression in Systemic Lupus Erythematosus Patients

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Background: *Systemic lupus erythematosus (SLE) is an autoimmune disease. The cause of the disease is related to multiple factors and involves immune responses. To the best of our knowledge, the relationship between the production of interleukin-2 (IL-2) and Nck adaptor molecule gene expression by lymphocytes in patients with SLE has not been studied.*
Objective: *Assess the association between IL-2 levels and Nck mRNA gene expression in CD3/CD28 and PHA/PMA stimulated peripheral blood mononuclear cells (PBMCs) from SLE patients compared with those from healthy donors.*
Material and Method: *The present study included 35 SLE patients and six healthy blood donors. Blood samples from these subjects were isolated to obtain PBMCs. Then Nck mRNA gene expression from these cells were quantitatively assessed using real-time reverse-transcriptase polymerase chain reaction (real-time RT-PCR). After stimulation of the PBMCs either with CD3/CD28 antibodies or PHA/PMA, IL-2 production and CD69 expression were assessed by enzyme-linked immunosorbent assay (ELISA) and flow cytometry, respectively.*
Results: *First, a defect in IL-2 levels was not observed in either CD3/CD28 or PHA/PMA stimulated PBMCs when compared with controls. Second, there was no difference in the Nck1 and Nck2 mRNA expression between the SLE patients and the healthy subjects.*
Conclusion: *We found that in SLE patients lymphocyte production of IL-2 did not decrease when compared with that of normal subjects. The Nck1 and Nck2 mRNA expression was not defective in SLE patients. We did not see any altered relation between IL-2 levels and Nck1 and Nck2 gene expression.*

Keywords: *IL-2, Nck, SLE*

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Systemic lupus erythematosus (SLE) is an autoimmune disorder predominantly found in women in reproductive years. The cause of the disease is related to multiple factors and involved the innate and adaptive immune responses[1-7]. In adaptive immunity, T cell abnormalities found in SLE include those involving T cell receptors, enzymes, and intracellular signaling[1,3,5,6,8-18]. The production of cytokines by T cells in SLE patients is affected especially interleukin-2 (IL-2) production, resulting in abnormal function of CD4 T cells that involves induction and maintenance of T cell homeostasis[19,20]. The association and expression levels of IL-2 with SLE immunopathologies have previously been reported[10,21,22].

The non-catalytic region of tyrosine kinase (Nck) is a type of adaptive proteins that functions in T cell signaling[23-30]. Nck protein has two isoforms, Nck1 and Nck2. Each contains three N-terminal Src homology (SH) 3 domains and a single C-terminal SH2 domain[31]. Nck has a role in stimulation of T cell receptor-CD3 (TCR) complex and production of IL-2. When TCR-CD3 complex is stimulated, Nck binds to the cytoplasmic tail of CD3ε[24]. This leads to production of many cytokines, especially IL-2. In 2010, Roy et al found that Nck deletion resulted in a severe T-cell lymphopenia and Nck adaptor protein is required for optimal T cell activation[27]. Recently, Yiemwattana et al found that reduced Nck1 expression lead to an impairment of TCR-CD3 complex activation and decreased IL-2 secretion[30].

To the best of our knowledge, an association between IL-2 molecule and Nck gene expression in SLE patients has not been studied. Therefore, the aim of the present study was to assess an association between IL-2 level and Nck mRNA expression in SLE patients.

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Material and Method

Study subjects

The present study was an experimental study. Thirty-five SLE patients and six healthy donors who donated blood at blood bank in Naresuan University Hospital were studied. The patients were recruited between 2013 and 2014 in Naresuan University Hospital, Phitsanulok, Thailand. All patients met four or more of the ACR criteria for the classification of SLE. Medical history was taken and physical examination was conducted by two rheumatologists. Laboratory tests were requested if needed. Patients on medications that included steroid and other medications were included. Patients on immunosuppressive drugs, cyclosporine, or tacrolimus were excluded. Patients with infection, having CRP of more than 10 mg/dL were also excluded. The SLE disease activity index (SLEDAI) was used to evaluate the disease activity. This present study was approved by the Research Ethics Committee of Naresuan University, (IRB No. 029/56). Written informed consents were obtained from all subjects. After obtaining informed consent, a 20-milliliters of venous blood sample was taken from each participant and the checklist consisting of age, sex, SLEDAI was completed. Fig. 1 showed a diagram of the study method.

Isolation of PBMCs

PBMCs were isolated from the venous whole blood obtained from all subjects. Then, approximately 10^7 cells were taken to select CD4 T cells using a MACs CD4+ Cell Isolation Kit II (Miltenyi Biotec GmbH, Bergisch Gladbach, Germany). The purity of the CD4 T cells was greater than 90% as measured by flow cytometry. These isolated CD4 T cells were then subjected to total RNA isolation for quantitation of Nck1 and Nck2 mRNA, and the remaining PBMCs were subject to cell stimulation (see below).

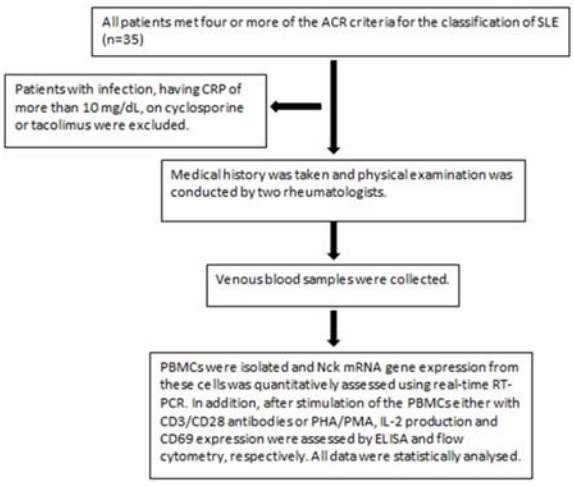

Fig. 1 A diagram of the study method.

Cell stimulation

The remaining PBMCs were plated onto 96-well tissue culture plates at a concentration of $1x10^5$ cells/well. They were either stimulated with: (1) 10 mg/ml anti-CD3 monoclonal antibody (mAb) plus 10 g/ml anti-CD28 mAb (eBioscience, San Diego, CA, USA), (2) 6 mg/ml PHA plus 1 ng/ml PMA (Sigma Aldrich, St. Louis, MO, USA) for 24 hours, or (3) none.

Quantitative reverse-transcriptase PCR

To quantitate the absolute mRNA concentration of Nck1 and Nck2, total RNA was isolated from PBMCs using Micro-to-Midi Total RNA purification kit (Invitrogen, Carlsbad, CA, USA). Fluorophore-labeled LUX primers and their unlabeled counterpart oligonucleotides were designed and supplied by Invitrogen. For qPCR reaction, the first-strand cDNAs were synthesized and amplified using the SuperScript III Platinum One-Step qRT-PCR system (Invitrogen) according to the manufacturer's instruction. The reaction was carried out in a spectrofluorometric thermal cycler (Roter-Gene 300, Corbett Research, Sydney, Australia).

IL-2 ELISA

Secreted IL-2 in the supernatant of cultured PBMCs was determined using a commercial enzyme-linked immunosorbent assay (ELISA) kit (R&D, Minneapolis, MN, USA) following the manufacturer's instructions. The optical density at 450 nm was read using a microplate reader (Perkin Elmer Life Sciences, IL, USA).

CD69 Flow cytometry

To determine the expression of CD69, the cultured PBMCs were stained with phycoerythrin-conjugated anti-CD69 antibody (eBioscience, San Diego, CA, USA) and analyzed on a FACSCalibur (Becton Dickenson, NJ, USA) and data were analyzed with CellQuestPro software.

Statistical analysis

All data were analyzed by using a statistical analysis software package (Minitab14). The statistics were used for percentage, mean, standard deviation

(SD) and independent sample t-test. Demographic data were analyzed by descriptive statistics. A *p*-value of <0.05 was considered statistically significant.

Results

The present study included a total of 35 SLE patients and six healthy donors. The PBMCs were collected to investigate the Nck1 and Nck2 mRNA expression in CD4 T cells as well as IL-2 production. Most patients were female in reproductive age (94.3%), and 51.4% of the patients had non-active disease. Most patients with active SLE disease had mild disease activity. Characteristic data of SLE patients were summarized in Table 1.

The present study showed that CD4 T cells from all subjects expressed significant more numbers of Nck1 than Nck2 mRNA. However, both Nck1 and Nck2 mRNA gene expression was not significant difference among SLE patients and healthy donors (Fig. 2A&B).

In addition, PBMCs from SLE patients and healthy donors stimulated by either anti-CD3/anti-CD28 antibodies or PHA/PMA produced similar elevated levels of IL-2 (Fig. 3). Similarly, there were comparable numbers of CD69-positive PBMCs from the patients and healthy donors stimulated by both anti-CD3/anti-CD28 antibodies and PHA/PMA (data not shown).

The present study included a total of 35 SLE patients. Blood samples from 35 patients were collected to assess IL-2 production in stimulated PBMCs. Characteristic data of SLE patients were shown in Table 1. First, this study examined the level IL-2 in SLE. When PBMCs of SLE patients were stimulated by CD3/CD28 antibodies and PHA/PMA, IL-2 levels were not significantly different from normal control. The IL-2 cytokine levels did not correlate with clinical or serologic activity of the disease. Second, expression of Nck1 and Nck2 mRNA by CD4 T cells did not significantly differ between SLE patients and normal donors. We assumed that there was no alteration in the relation between IL-2 cytokine production and Nck1 as well as Nck2 mRNA expression.

Discussion

SLE is a human chronic autoimmune disease characterized by disordered immune regulation. The activation of T cells or B cells is often recognized in SLE patients. IL-2 is essential for both promotion and suppression of the immune response. This cytokine constitutes a key element in the maintenance of the

Table 1. Characteristic data of SLE patients

Characteristic data	SLE, n (%) Total = 35 cases
Age (years, mean ± SD)	33.51 (±14.86)
Female (percent)	33 (94.3)
Hemoglobin (g/dl) (mean ± SD)	12.04 (±1.90)
Hematocrit (%) (mean ± SD)	35.9 (±5.05)
White blood cell (cell/µL) (mean ± SD)	6,583 (±2,338)
Total lymphocyte (cell/µL) (mean ± SD)	1,663 (±872)
Platelet (cell/µL) (mean ± SD)	284,228 (±125,530)
Creatinine (mg%) (mean ± SD)	0.74 (±0.42)
24 hours urine protein (mg) (mean ± SD)	476.64 (±817.14)
24 hours urine creatinine (g) (mean ± SD)	0.85 (±0.40)
Urine protein creatinine index (mean ± SD)	0.90 (±1.55)
C-reactive protein (mg/dL)	3.35 (±4.03)
Serum albumin (g/dl) (mean ± SD)	4.12 (±0.46)
C3 complement (mg/dl) (mean ± SD)	91.97 (±28.98)
C4 complement (mg/dl) (mean ± SD)	22.86 (±11.08)
CH50 (U/mL) (mean ± SD)	27.34 (±9.6)
SLEDAI	
No active (SLEDAI = 0) frequency (percent)	18 (51.4)
Mild activity (SLEDAI = 1-5) frequency (percent)	13 (37.1)
Moderate activity (SLEDAI = 6-10) frequency (percent)	2 (5.7)
High activity (SLEDAI = 11-19) frequency (percent)	2 (5.7)

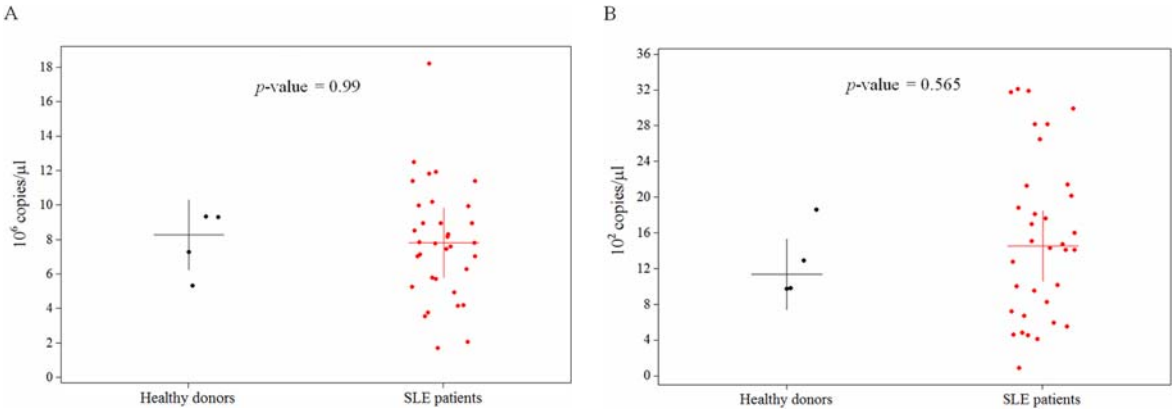

Fig. 2 PBMCs were assessed for Nck1 and Nck2 mRNA expression using real-time RT-PCR. Nck1 (A) and Nck2 (B) mRNA gene expression in CD4 T cells detected using real-time quantitative RT-PCR. The data are shown as a dot-plot of a copy number of the Nck genes per µl from individual subjects. The horizontal lines are the median value of each group.

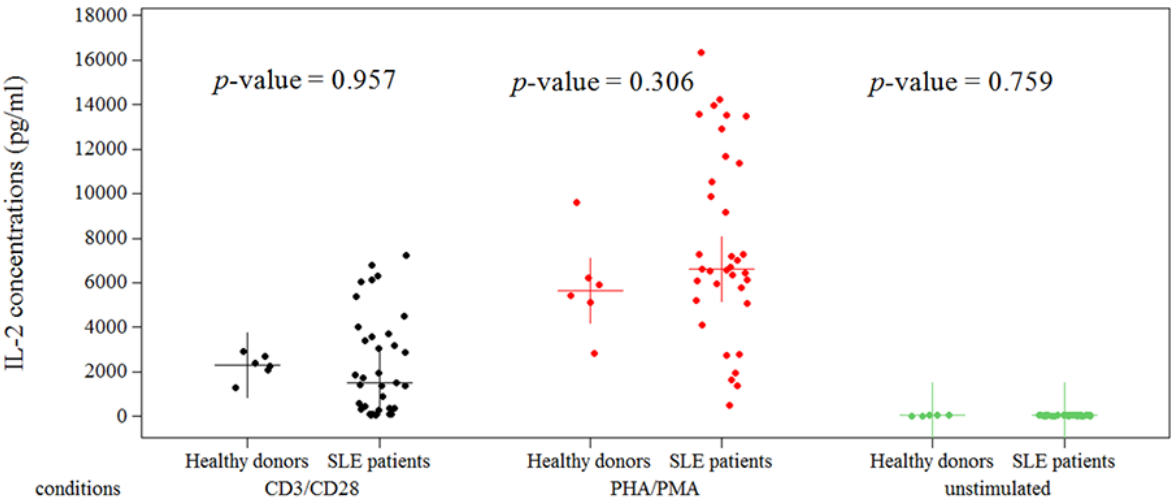

Fig. 3 PBMCs were stimulated with CD3/CD28 antibodies or PHA/PMA or none. The data are shown as a dot-plot of IL-2 concentrations in pg/ml of individual subject PBMCs that were cultured in the indicated conditions. The horizontal lines are the median value of each group.

homeostasis between a proliferative immune response and the induction of tolerance[10]. In SLE, previous reports had shown that T cells from patients and mice produce decreased amounts of IL-2, which may involve in increased susceptibility to infection and subsequently extended survival of autoreactive lymphocyte[13].

In 1981, deficient IL-2 activity was shown in diverse lupus murine models with different background[32]. Many defects associated with decreased IL-2 production in SLE T cells were involved in the transcriptional repression of IL-2 in SLE[13]. In contrast, another report had shown that the presence of deficient IL-2 production by T cells in SLE patients was related to disease activity[33]. In these studies, all patients were in active disease and none of them received corticosteroids, antimalarial or immunosuppressive drug. In our study, although all patients received corticosteroids, antimalarial drug and some patients were in inactive disease, they had normal production of IL-2 in their stimulated PBMCs.

To the best of our knowledge, our study is the first to demonstrate the mRNA expression levels of Nck1 and Nck2 in SLE patients compared to healthy subjects. We did not observe any difference in Nck1 and Nck2 mRNA expression between SLE patients and

healthy subjects. Although these patients received corticosteroids, antimalarial and immunosuppressive drug, upon stimulation, PBMCs from both SLE patients and healthy donors produced similarly elevated levels of IL-2. For clinical application, the present study showed that the Nck1 and Nck2 mRNA expression was not defective in SLE patients and there was no alteration in the relationship between IL-2 levels and Nck1 and Nck2 gene expression. Therefore, an examination of IL-2 level and Nck1 and Nck2 gene expression may not needed in SLE patients. Medications that could be developed in the future to inhibit interaction between Nck molecule and TCR complex may be ineffective in SLE patients. Our present study had a limitation in that the sample size of SLE patients was small. However, we can conclude that the Nck1 and Nck2 gene expression in SLE patients do not differ from those in normal subjects, and thus, the Nck molecules may not be involved in abnormality of T cells in SLE.

Conclusion

Our study found that in SLE patients, IL-2 production by stimulated PBMCs were not different from those in healthy individuals. SLE patients also had normal levels of Nck1 and Nck2 mRNA gene expression in CD4 T cells compared to those of normal subjects. We assumed that there was no alteration in the relationship between IL-2 protein expression and Nck1 and Nck2 mRNA levels in these SLE patients.

What is already known on this topic?

In SLE, previous reports have shown that T cells from patients and mice produce decreased amounts of IL-2, which may involve in increased susceptibility to infection and subsequent extended survival of autoreactive lymphocyte[13]. Recently, Yiemwattana et al found that reduced Nck1 expression lead to an impairment of TCR-CD3 complex activation and decreased IL-2 secretion[30]. However, the data of previous studies had not included the link between IL-2 production and Nck adaptor molecule gene expression in SLE patients.

What this study adds?

To the best of our knowledge, our study is the first to demonstrate the mRNA expression levels of Nck1 and Nck2 in SLE patients compared to healthy subjects. In SLE patients, IL-2 production by stimulated PBMCs was not different from that in healthy individuals. SLE patients also had normal levels of Nck1 and Nck2 mRNA gene expression in CD4 T cells compared to those of normal subjects.

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Potential conflict of interest

None.

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การศึกษาระดับ Interleukin-2 และการแสดงออกของจีน Nck adaptor molecule ในผู้ป่วยโรคเอสแอลอี

ปฐพงศ์ โตวิวัฒน์, บดินทร์ บุตรธรรม

ภูมิหลัง: โรคเอสแอลอีจัดเป็นโรคที่มีความผิดปกติทางภูมิคุ้มกันชนิดหนึ่ง ซึ่งผู้ป่วยมีความหลากหลายในการแสดงอาการของโรค โดยพยาธิสภาพการเกิดโรคนั้นมีหลายปัจจัยและยังเกี่ยวข้องกับระบบภูมิคุ้มกันของร่างกาย อีกทั้งยังมีการเปลี่ยนแปลงของระดับสารน้ำทางชีวภาพมากมาย ซึ่ง IL-2 จัดเป็นสารน้ำทางชีวภาพที่มีการศึกษากันอย่างแพร่หลาย และในปัจจุบันพบว่ามีความสัมพันธ์ระหว่างระดับ IL-2 และการแสดงออกของจีน Nck mRNA แต่อย่างไรก็ตามยังไม่พบว่ามีการศึกษาถึงความสัมพันธ์ดังกล่าวในผู้ป่วยโรคเอสแอลอี

วัตถุประสงค์: เพื่อศึกษาความสัมพันธ์ระหว่างระดับ IL-2 และการแสดงออกของจีน Nck mRNA โดยการกระตุ้น CD3/CD28 และ PHA/PMA ของเม็ดเลือดขาวชนิด mononuclear cells ในผู้ป่วยโรคเอสเอลอีเทียบกับกลุ่มควบคุม

วัสดุและวิธีการ: ในการศึกษาครั้งนี้มีผู้ป่วยที่เป็นโรคเอสแอลอีที่เข้าร่วมโครงการวิจัยจำนวน 35 ราย และกลุ่มควบคุมซึ่งเป็นคนปกติจำนวน 6 ราย และเมื่อได้ทำการเจาะเลือดแล้ว ตัวอย่างเลือดถูกแยกเพื่อให้ได้เม็ดเลือดขาวชนิด mononuclear cells หลังจากนั้นวัดการแสดงออกของจีน Nck mRNA โดยใช้วิธีการ real-time reverse-transcriptase polymerase chain reaction (real-time RT-PCR) ต่อมาทำการกระตุ้นเม็ดเลือดขาวชนิด mononuclear cells ด้วยแอนติบอดีต่อ CD3/CD28 หรือ PHA/PMA แล้วตรวจวัดระดับ IL-2 ที่เกิดขึ้นและการแสดงออกของ CD69 โดยวิธี enzyme-linked immunosorbant assay (ELISA) และ flow cytometry ตามลำดับ

ผลการศึกษา: ผลการศึกษาประการแรก พบว่าเมื่อกระตุ้นเม็ดเลือดขาวชนิด mononuclear cells โดยการกระตุ้นด้วยแอนติบอดีต่อ CD3/CD28 หรือด้วยสาร PHA/PMA แล้วระดับ IL-2 ไม่มีความแตกต่างกันระหว่างกลุ่มผู้ป่วยที่เป็นโรคเอสแอลอีและกลุ่มควบคุมซึ่งเป็นคนปกติ ผลการศึกษาประการที่สองพบว่าการแสดงออกของจีน Nck1 and Nck2 mRNA ไม่มีความแตกต่างกันระหว่างกลุ่มผู้ป่วยที่เป็นโรคเอสแอลอี และกลุ่มควบคุมซึ่งเป็นคนปกติเช่นกัน

สรุป: การศึกษานี้พบว่าเม็ดเลือดขาวชนิด lymphocyte ในผู้ป่วยที่เป็นโรคเอสแอลอีสามารถผลิตสาร IL-2 ได้ไม่แตกต่างจากคนปกติ ส่วนการแสดงออกของจีน Nck1 และ Nck2 mRNA พบว่าไม่มีความผิดปกติในผู้ป่วยโรคเอสแอลอี และยังไม่พบว่ามีความสัมพันธ์ที่เปลี่ยนแปลงไประหว่างระดับ IL-2 และการแสดงออกของจีน Nck1 และ Nck2