



Pathological significance and regulatory mechanism of lymphotoxin β receptor overexpression in T cells of patients with systemic lupus erythematosus

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Abstract

Systemic lupus erythematosus (SLE) is a typical autoimmune disease. Lymphotoxin β receptor (LT β R) signaling plays an important role in autoimmune inflammations. LT β R-Ig fusion protein, LT β R blocking agent, has been used to treat SLE, while its mechanism remains to be fully elucidated. In this study, to investigate the expression of LT β R in the T cells of SLE patients and its roles in the pathogenesis of SLE, we isolated the peripheral blood T cells of SLE patients and normal controls to detect expression of LT β R by flow cytometry and RNA assay. T cells were also stimulated with LIGHT, a ligand of LT β R, and then detected for their LT β R expressions and apoptosis by flow cytometry. Also, their expressions of inflammatory factors and receptors were determined by RNA assay. The results showed that LT β R positive cells were 22.75% \pm 6.98% in CD3⁺ cells of SLE patients, while there were almost no LT β R positive cells in CD3⁺ cells of normal persons. Moreover, LT β R expression was remarkably higher in CD3, CD4 and CD8 positive T cells of active SLE patients than non/low active patients (all $P < 0.05$), and positively correlated with increased Ig level, decreased complement level and renal damage. Moreover, the stimulation of SLE T cells with LIGHT promoted higher expression of LT β R, IL-23R and IL-17A, and apoptosis of T cells. In conclusion, we demonstrated a high expression of LT β R in the T cells of SLE patients which may be associated with pathogenesis of SLE.

Keywords: lupus erythematosus, systemic (SLE), lymphotoxin β receptor (LT β R), interleukin-17 (IL-17), interleukin-23 receptor (IL-23R)

Introduction

Systemic lupus erythematosus (SLE) is a typical autoimmune disease. The mechanism of SLE pathogenesis is related to autoimmune antibody production,

abnormal activation and apoptosis of T cells, and autoimmune inflammation, which is not fully elucidated. Lymphotoxin (LT) is one part of signaling molecule network responsible for connection of lymphocytes and the internal environment. LT has two

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putative subunits, LT α and LT β . The main active form is the heterotrimer LT α 1 β 2 composed of one LT α molecule and two LT β molecules^[1]. LT β receptor (LT β R) combines with the ligands, LT and LIGHT, a LT-related inducible ligand that competes for glycoprotein D binding to herpesvirus entry mediator on T cells. LT β R signaling plays an important role in the occurrence and development of peripheral lymphoid tissue. Mice lacking LT α , LT β or LT β R lack the development of secondary lymphoid tissue and the homeostasis of the internal environment of the lymphatic tissue is destroyed. LT β R signaling is also connected with some specific inflammatory chemokines, interferon and so on, being of great significance in autoimmunity and anti-infection^[2-3]. It leads to the activation of NF- κ B signaling through TRAF2/3, and then initiates expressions of signaling inflammatory genes^[4]. The LT/LIGHT signaling pathway of T cells may mediate inflammation in autoimmune and infectious diseases. Studies have shown that LT β R signaling is related with autoimmune diseases^[5]. Target-elimination of LT α (LT β R ligand functional subunit) expressed in Th1 and Th17 cells suppressed autoimmune diseases^[6]. Other studies have shown that LT β R mainly exists on the surface of monocyte-macrophages and endothelial cells. LT α 1 β 2/LIGHT-LT β R can induce expressions of pro-inflammatory genes of vascular endothelial cells depending on the classical and non-classical NF- κ B pathway. Some researchers have used LT β R-Ig fusion protein, LT β R blocking agent, for the experimental treatment of collagen induced arthritis^[7], experimental allergic encephalomyelitis (EAE)^[8], T cell mediated liver injury^[9] and other animal models of autoimmune diseases, which can alleviate autoimmune inflammation. There are also some studies that try to use this method for treating SLE^[10]. However, the exact mechanism remains to be further elucidated.

Recently, it has been found that Th-17 cells play an important role in SLE, promoting the formation of inflammatory environment and the activation and development of autoimmune response^[11-13]. Studies also showed that IL-23R was highly expressed by T cells, which was positively correlated with IL-17 expression, in SLE patients^[14-18].

Our previous studies found that the apoptosis of SLE T cell subsets was increased and the Fas/FasL pathway was involved in the mechanism of apoptosis. In SLE patients, the increase of T cell apoptosis was positively correlated with the increased expression of Fas/FasL on the membrane surface of T cell subsets. The abnormal high level of IL-10 in the serum of SLE patients could induce T cells, especially CD4⁺ T cells, to express Fas/

FasL highly and promote apoptosis of T cells. However, blocking the Fas-FasL pathway by using FasL antibody partly, rather than completely, inhibited the apoptosis of T cells^[19-20]. On the other hand, the effect of Fas/FasL signaling induced by SLE serum IL-10 on the apoptosis of CD8⁺ T cells was relatively small^[21]. These results suggest that, besides Fas/FasL, there might be other mechanisms of T cell apoptosis in SLE patients.

It is well known that LT α 1 β 2/LIGHT secreted by T cells combines with the receptor, LT β R, on the surface of monocyte-macrophages and endothelial cells, resulting in the expressions of inflammatory factors by these cells. However, how T cells interact by LT α 1 β 2/LIGHT-LT β R signaling under pathological condition, for example in SLE remain to be elucidated and the effects of LT β R signaling on the expressions of inflammatory factors by T cells and on the apoptosis of T cells in SLE also require to be characterized. In this study, we investigated the expression of LT β R in the T cells of SLE patients and its effects on expression of inflammation-related factors and apoptosis of SLE T cells.

Subjects and methods

Subjects

The peripheral blood samples from 247 patients with SLE, including 189 females and 58 males with a mean age of 28.5 years, were collected. These patients were inpatients of Rheumatology Department and Nephrology Department of the First Affiliated Hospital of Nanjing Medical University and Rheumatology Department of Nanjing First Hospital and fulfilled the revised criteria of American College of Rheumatology for SLE. Totally 247 healthy blood donors matched in age and sex were used as the normal control. The study was approved by the Research Ethics Committee of Nanjing Medical University. Informed consent was obtained from all patients and control subjects.

Magnetic bead sorting T cells

The peripheral blood mononuclear cells of SLE patients and healthy blood donors were separated by density gradient centrifugation with Ficoll-histopaque 1077 (Sigma, St. Louis, MO, USA) and human CD3⁺ T cell positive sorting mini-magnetic beads (20 μ L for 1 \times 10⁷ cells) were added. Then, they were incubated at 4°C for 15 minutes and washed, and then CD3⁺ T cells were separated with magnetic cell sorting column (MS positive sorting column) and magnetic cell separator. The beads, the column and the separator were all from Miltenyi (Germany).

T cell culture and treatment

CD3⁺ T cells of SLE patients and healthy donors at a density of 1×10^6 /mL were cultured in complete 1640 medium in the following 4 groups: the phorbol-12-myristate-13-acetate (PMA, 100 ng/mL, Sigma) group, dexamethasone (DEX, 10 nmol/mL, Sigma Co., USA) group, LIGHT (3 μ g/mL, Perotech Co., USA) group and control group (1640 medium) for 24 and 48 hours.

RT-PCR

Using Trizo, total cellular RNA of dissolved CD3⁺ T cells were extracted and then reverse-transcribed to cDNA with a reverse-transcription kit (Bori Biological Products Co. Ltd, Hangzhou, China) according to the kit instructions. Using the cDNA, specific DNA fragments of target genes were amplified by PCR with the primer sequences shown in **Table 1**. These primers were purchased from Invitrogen Life Technology (USA). The PCR protocol was as follows: pre-incubation step at 94°C for 2 minutes, followed by amplification for 30 cycles of 94°C for 40 seconds, 52°C (LT- β R) or 53.2°C (IL-17A) or 52.4°C (IL-23R) for 45 seconds and 72°C for 60 seconds, and a final extension step at 72°C for 5 minutes.

Gene chip

Extracted total RNA of CD3⁺ T cells were sent to Shanghai Kangcheng Biotech Co. (China) for analysis of RNA expressions with gene chip and sequencing.

Flow cytometry and fluorescent microscopy

After staining cells with FITC-antibody to CD3, CD3⁺ cell purity was detected by flow cytometry with a flow cytometer (FACScan, BD Co., USA). After staining cells with antibodies of LT β R-PE, CD3-APC, CD8-FITC and their isotype controls (all from Ebioscience, USA), LT β R expression on T cell membrane was detected by flow cytometry and also with a fluorescent microscopy (Carl Zeiss Jena Co., Germany).

Flow cytometry was also used for detection of CD3⁺ T cell apoptosis using Annexin V kit (Ebioscience) and for detection of cell cycle after treating T cells with propidium iodide (PI) and RNase (Yingjun Biological Co., Shanghai, China).

ELISA

Serum IL-6 levels of SLE patients were determined by ELIAS with the IL-6 ELISA kit (BD Co., USA).

Statistical analysis

The data were input with Adopt Microsoft Office Excel Software (2007) to establish a database. SPSS version 18.0 (SPSS Inc., Chicago, IL, USA) was used for statistical analysis. The data of LT β R expressions of the SLE and normal control groups were shown as mean \pm standard deviation ($\bar{x} \pm SD$). The two groups were compared using *t* test or analysis of variance. Multiple comparisons between groups were performed using the SNK method. Correlation between two variables was used for linear correlation analysis. $P < 0.05$ was considered to be statistically significant.

Results

High expression of LT β R on CD3⁺ T cells of SLE patients and its positive correlation with disease activity

The purity of CD3⁺ T cells freshly isolated with magnetic beads reached $95.36\% \pm 3.47\%$. The gene chip results showed that LT β R was abnormally highly expressed in the T cells of SLE patients (**Fig. 1A**). The results of RT-PCR also showed that LT β R mRNA was highly expressed in T cells of SLE patients, while almost undetectable in T cells of healthy blood donors (**Fig. 1B**). Then, we used flow cytometry to verify the gene chip results at the protein expression level. LT β R positive cells were $22.75\% \pm 6.98\%$ in CD3⁺ cells of SLE patients, while there were almost no LT β R positive cells in CD3⁺ cells of normal persons, and the difference was remarkable. The expressions of LT β R in the subgroups of CD4⁺T and CD8⁺T cells in SLE patients were both increased (**Fig. 2A** and **B**). The expression of LT β R on the surface of CD8⁺ T cells of SLE patients, rather than of normal persons, was proved by fluorescent microscopy (**Fig. 2C**).

Then, we analyzed the correlation between the expression of LT β R and disease activity of SLE patients. The expressions of LT β R were remarkably higher in CD3⁺ T cells, and in CD8 or CD4 positive T

Table 1 Primer sequences of amplified fragments of target genes used in PCR

Target gene	Upstream primer	Downstream primer	Size of amplified fragment
LT β R	catccatacttcctgact	cttctgcctttctcagact	481 bp
IL-17A	cctcttcctcctattctct	ctttgctgggtagtgaaagta	557 bp
IL-23R	caggtcctatattgtgatcc	tgaattcacactgaaacag	342 bp
β -actin	tcggcgccctgtagaggac	cggggagggaaggacgagg	268 bp

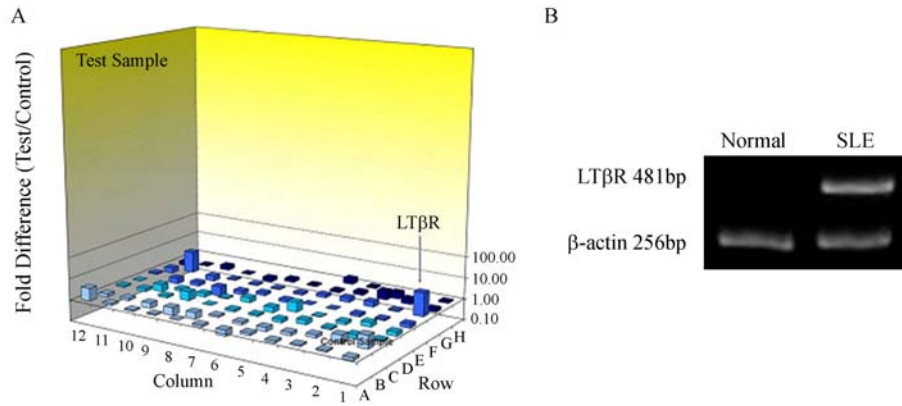


Fig. 1 The results of gene chip for apoptosis associated gene expressions and RT-PCR for *LTβR* mRNA. A: The results of gene chip for the expressions of apoptosis related genes, using RNA extracts T cells from SLE patients; B: Expressions of *LTβR* mRNA in CD3 positive T cells of healthy blood donors and SLE patients determined by RT-PCR.

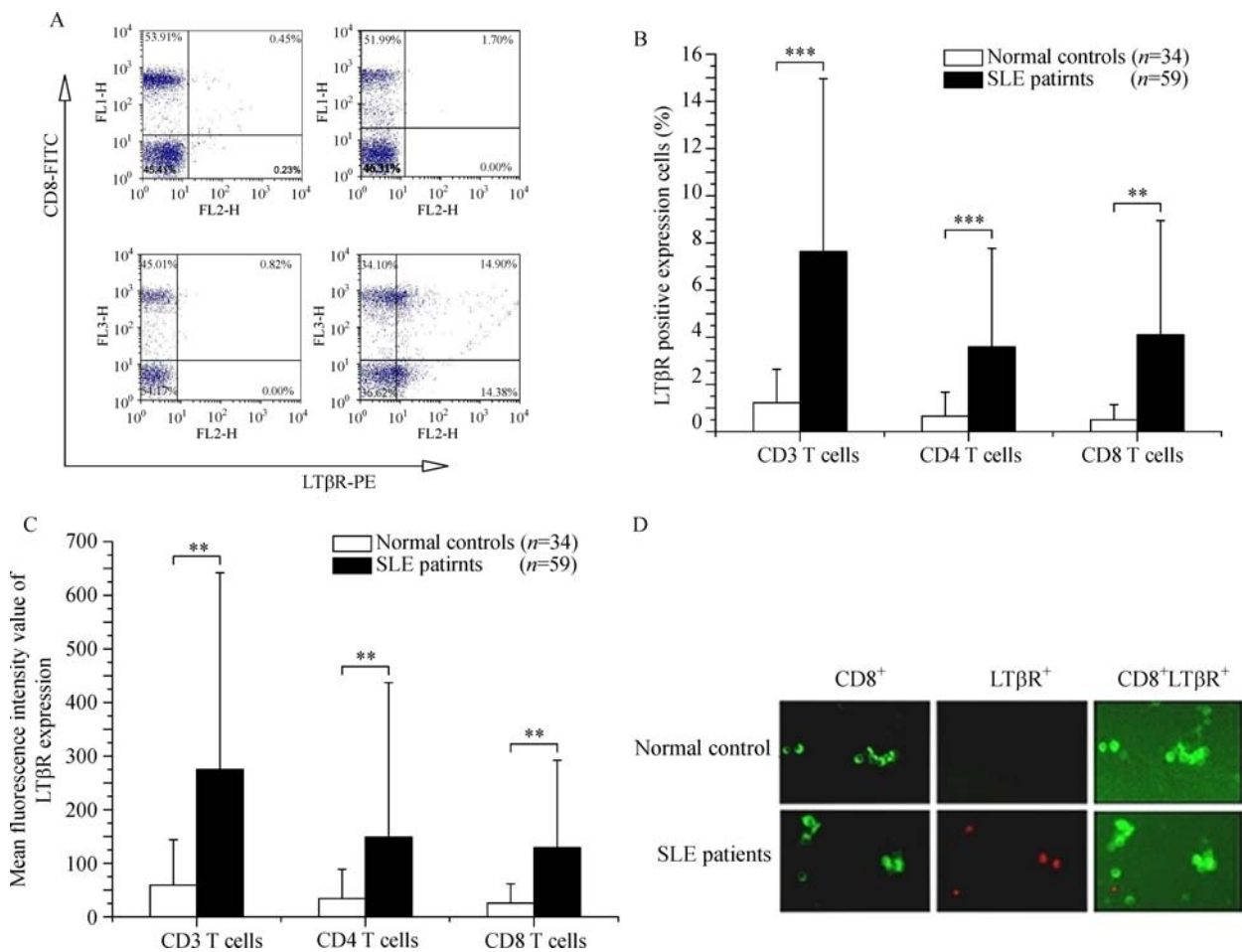


Fig. 2 *LTβR* expressions on the surface of T cells from SLE patients and normal persons. A to C: Results of flow cytometry for *LTβR* expressions on the surfaces of T cells from SLE patients and normal persons; D: The expression of *LTβR* in CD8⁺T cells from a patient with SLE, tested with fluorescent microscopy (FITC- antibody to CD8 showed green, PE-antibody to *LTβR* red). ***P*<0.01,****P*<0.001.

cells, of active SLE patients, compared with those of normal persons, rheumatoid arthritis (RA) patients and non/low active SLE patients (**Fig. 3A** and **3B**). At the mRNA level, the expression of *LTβR* in SLE patients

with high activity (SLEDAI≥6) was remarkably higher than that of patients with non/low activity(SLEDAI < 6) (**Fig. 3C**). These results suggested that the expression of *LTβR* was correlated positively with disease activity. To

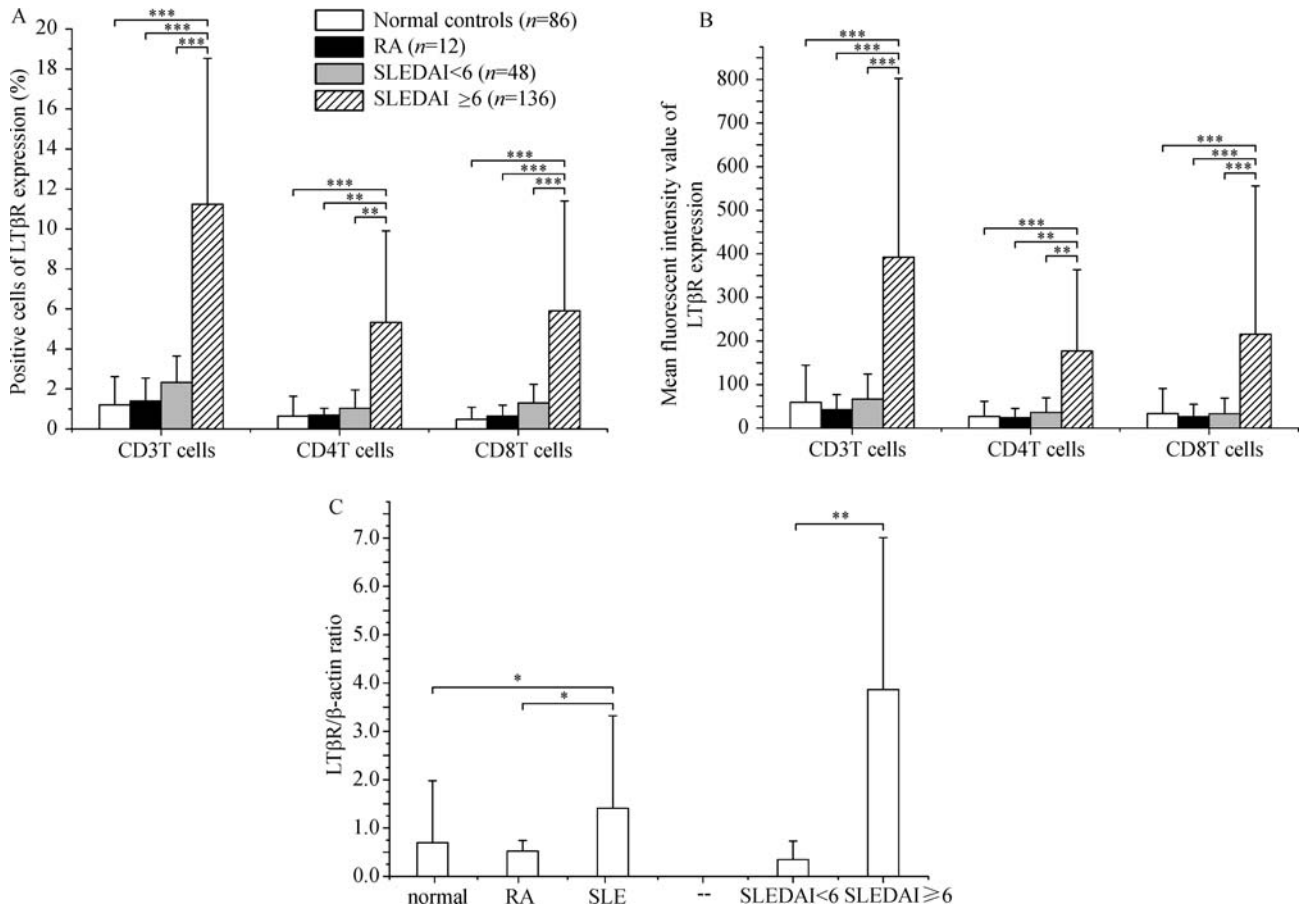


Fig. 3 Percentages of LTβR positivity in T cells from active, non/low active SLE patients, RA patients and control persons. A: Percentages of LTβR positive cells in CD3⁺T cell subgroups determined by flow cytometry; B: Fluorescent intensity values of LTβR. stained; C: LTβR mRNA expression in CD3⁺T cells tested by RT-PCR. * $P < 0.5$, ** $P < 0.01$, *** $P < 0.001$.

further understand the relationship between LTβR expression and clinical features, we grouped SLE patients based on different clinical features. There existed significant difference of LTβR expression, no matter by the percentages of positive cells or by fluorescent intensity, between the two groups of patients with increased and normal serum immunoglobulin levels, or of patients with decreased and normal serum complement levels, or between the two groups of patients with and without renal damage (**Fig. 4**).

LIGHT stimulation increased the expression of LTβR and the apoptosis of SLE patients' T cells in vitro

CD3⁺ T cells of SLE patients and normal persons were stimulated by PMA, dexamethasone (DEX) or LIGHT separately and cultured for 24 and 48 hours. Then, the T cells were analyzed by flow cytometry. The results showed that stimulation of LIGHT increased, while DEX decreased, the expression of LTβR in SLE patients' T cell subsets, but has no effect on normal persons' T cells (**Fig. 5**). Furthermore, the apoptosis of CD3⁺ T cells in SLE patients was significantly increased after stimulation with LIGHT, compared

with that in normal persons (**Fig. 6**). Cell cycle analysis showed that after stimulation and culture with LIGHT for 24 hours and 48 hours, the numbers of CD3⁺ T cells in G1 phase in SLE patients were 60.85 ± 8.01 and 76.77 ± 3.10 , respectively, while those in normal persons were 49.17 ± 2.17 and 56.22 ± 4.86 , respectively, the differences were statistically significant (both $P < 0.05$); the numbers of CD3⁺ T cells in M phase in SLE patients were 10.75 ± 3.12 and 8.18 ± 6.12 respectively, while those in normal persons were 16.78 ± 4.15 and 17.14 ± 8.18 , respectively, the differences were also significant (both $P < 0.05$); but there were no statistically significant differences between the numbers of CD3⁺ T cells in G0 and S phases in SLE patients and in normal persons.

LIGHT-LTβR signaling upregulated the expressions of IL-23R and IL-17A in SLE patients' T cells

The total cellular RNA extracted from CD3⁺ T cells of SLE patients was analyzed for expressions of inflammatory factor and their receptor related genes by gene chip assay. We found that CD3⁺T cells of SLE patients highly expressed IL-23R (**Fig. 7A**), which was proved by the RT-PCR results. We also detected IL-17A

mRNA simultaneously and found that CD3⁺T cells of SLE patients highly expressed IL-17A (Fig. 7B). Furthermore, the results of RT-PCR showed that stimulation of SLE patients' T cells with LIGHT also increased the mRNA levels of IL-23R and IL-17A in T cells (Fig. 8).

Positive correlation of the high expression of LTβR of T cells with serum levels of IL-6 in SLE patients

We use the ELISA method to detect IL-6 levels in the serum of SLE patients. The results showed that the

expression of LTβR was positively correlated with the level of IL-6 in SLE patients (Fig. 9).

Discussion

Previous studies suggested that LTβR was mainly expressed on the surfaces of fibroblast cells, epithelial cells and bone marrow cells (monocytes, dendritic cells, and mast cells). Here, our results proved that LTβR abnormally highly expressed in CD3⁺ T cells of SLE patients. The expression of LTβR was also correlated

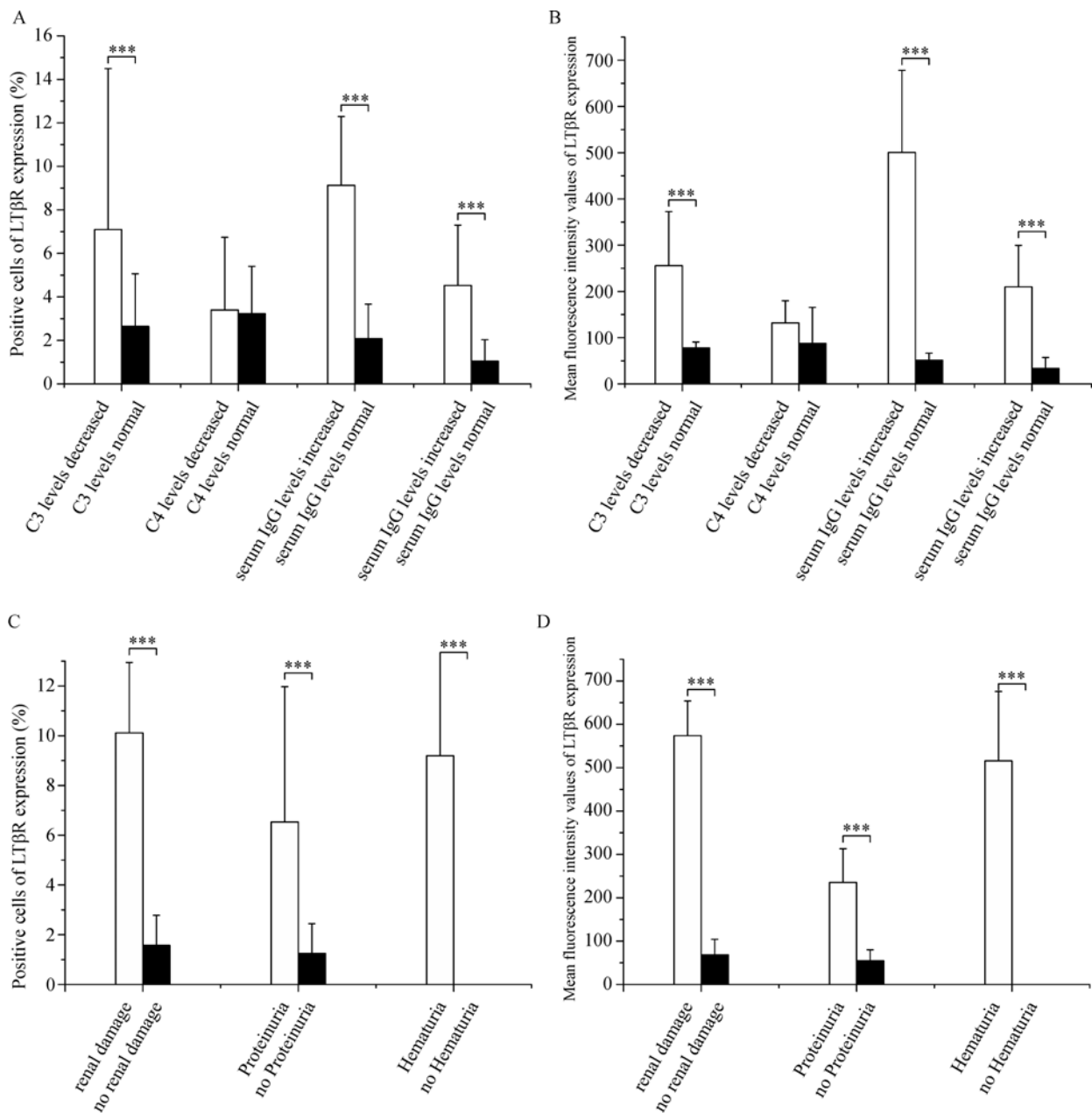


Fig. 4 The relationship between LTβR expression and clinical manifestations of SLE patients. (A) Positive rates and (B) mean fluorescence intensity values of LTβR expressions in T cells from SLE patients with normal and decreased levels of serum complement and with normal and increased levels of serum immunoglobulin; (C) Positive rates and (D) mean fluorescence intensity values of LTβR expressions in T cells from SLE patients with and without renal damage. ***P<0.001.

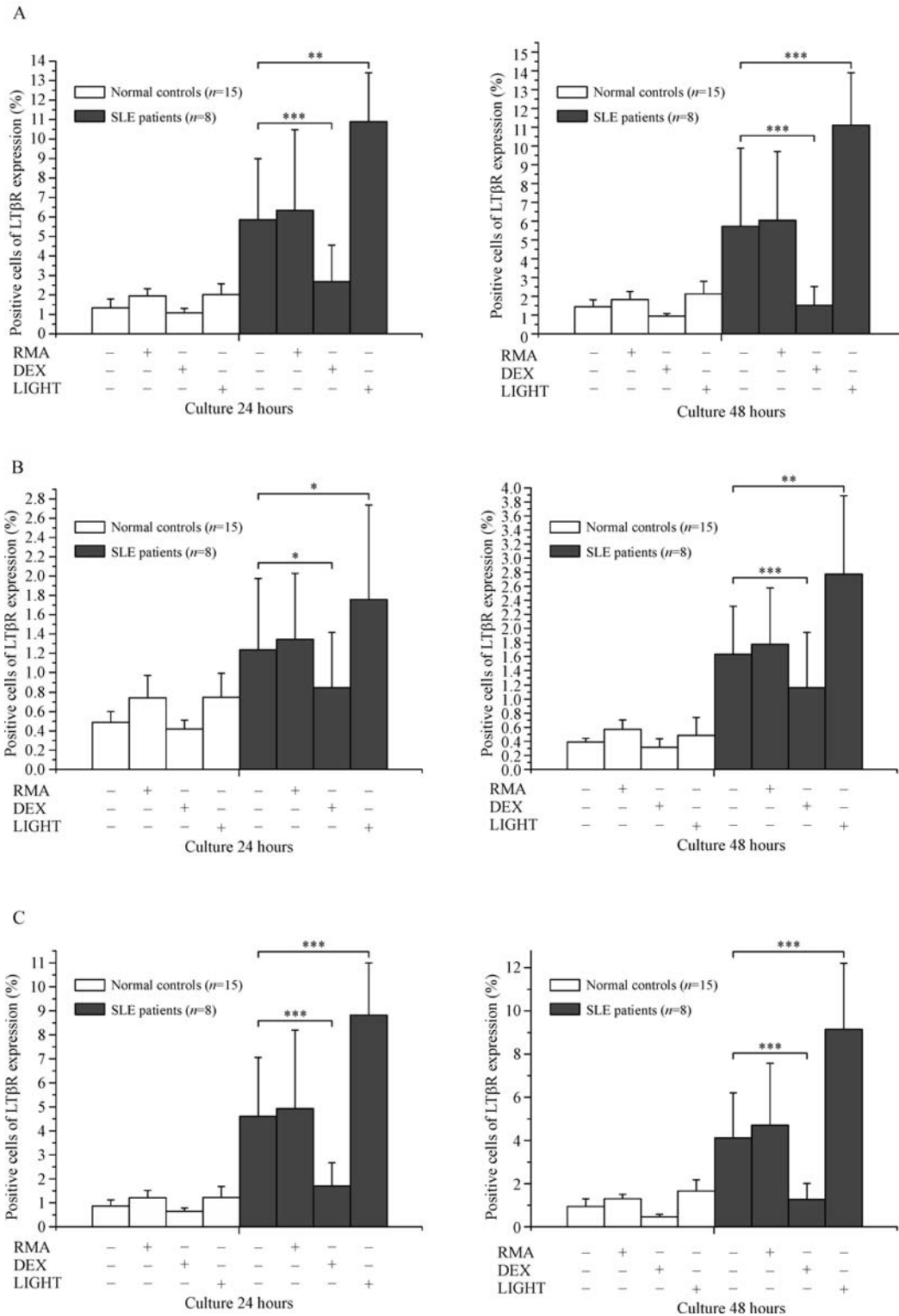


Fig. 5 Effect of LIGHT, DEX and PHA on LTβR expressions by CD3⁺ T cell subsets of SLE patients and normal persons. A: Positive cells of LTβR expression in CD3⁺ T cells; B: Positive cells of LTβR expression in CD4⁺ T cells; C: Positive cells of LTβR expression in CD8⁺ T cells. **P*<0.5, ***P*<0.01, ****P*<0.001.

positively with disease activity. We hypothesized that the abnormal expression of LTβR in SLE patients' T cells might have pathological significance.

In the presents study, we proved that LIGHT- LTβR

signaling promoted LTβR expression by T cells, forming a positive feedback. Secondly, we proved that LIGHT- LTβR signaling also promoted apoptosis of T cells of SLE patients, which might be another cause of T

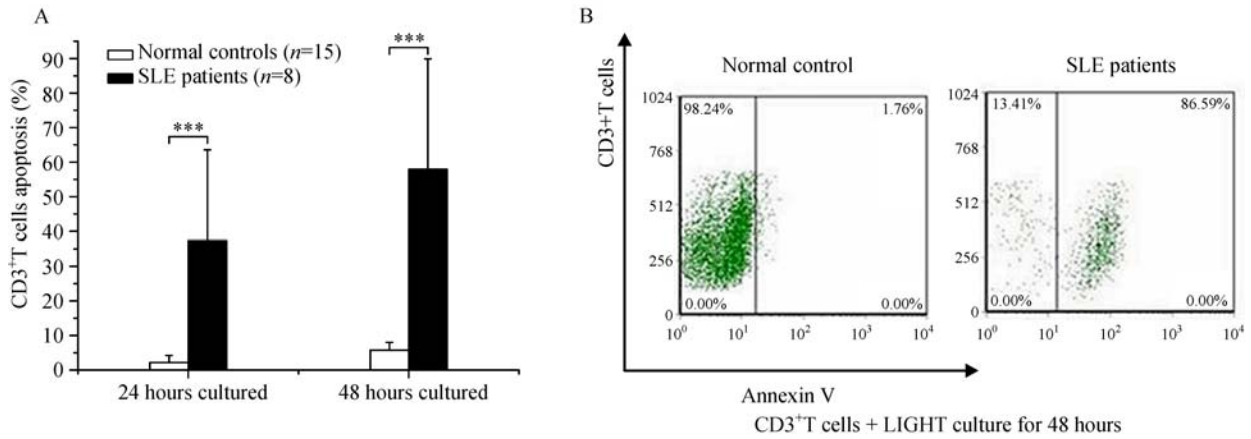


Fig. 6 Apoptosis of peripheral blood CD3⁺ T cells from SLE patients and normal persons after LIGHT treatment in vitro. A: Effect of LIGHT treatment on apoptosis of CD3⁺ T cells from normal persons and SLE patients; B: Flow cytometry scatter diagram, selected from one normal person and one SLE patient as the representative. ***P<0.001.

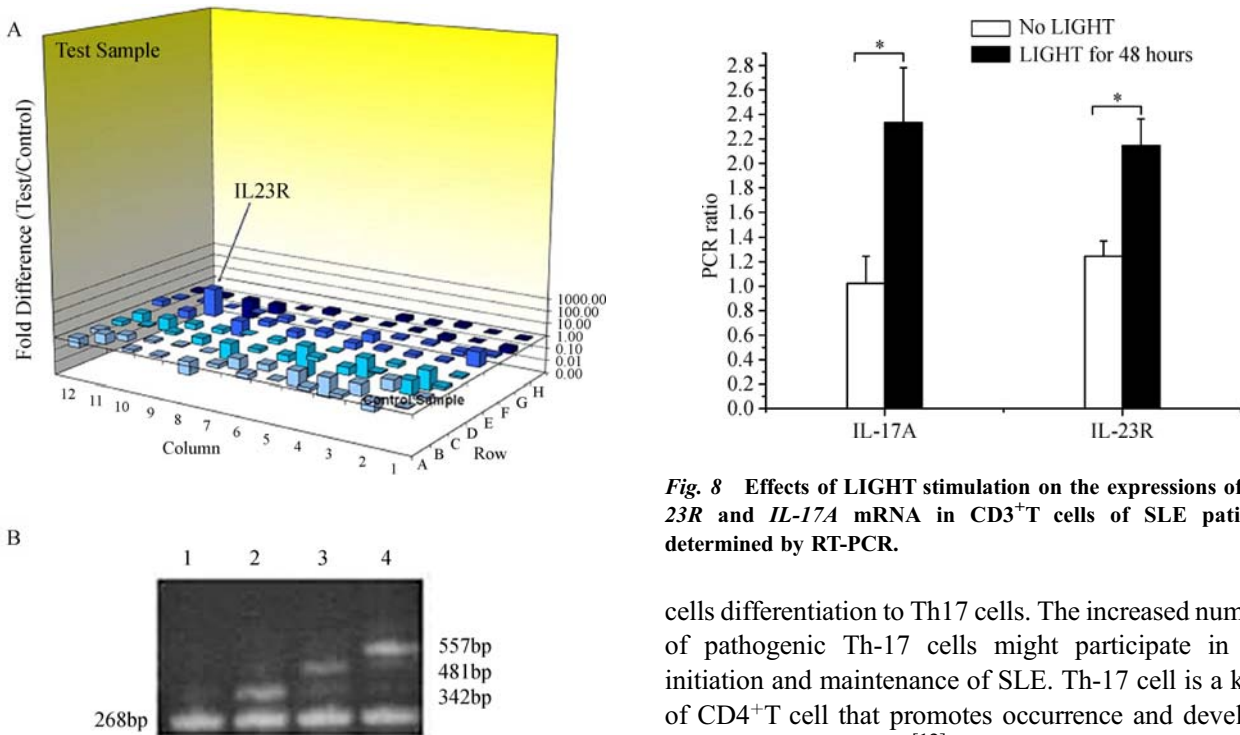


Fig. 7 Expressions of IL-23R and IL-17A by T cells from SLE patients. A: Gene chip showed a high expression of IL-23R in SLE patients; B: CD3⁺T cells from SLE patients (Lane 2,3 and 4), rather than normal person (Lane 1), could express LTβR (Lane 3, 481bp), IL-23R (Lane 2, 342bp) and IL-17A (Lane 4, 557bp) mRNA determined by RT-PCR. β-actin (268bp) was used as internal reference.

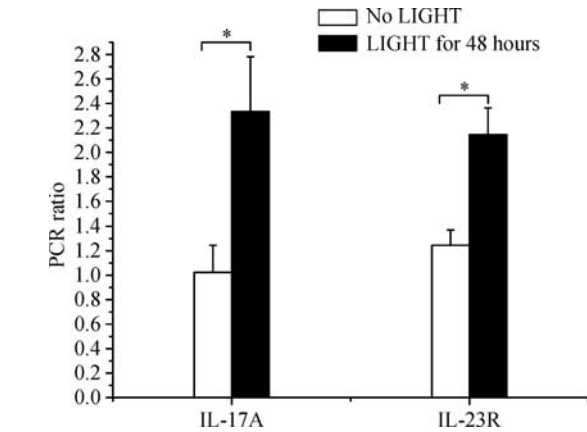


Fig. 8 Effects of LIGHT stimulation on the expressions of IL-23R and IL-17A mRNA in CD3⁺T cells of SLE patients determined by RT-PCR.

cell decrease. Thirdly, according to the above clues from the present study, we suggested that the enhancement of LTβR signaling in SLE patients' T cells promoted the expression of IL-23R and increased the sensitivity of T

cells differentiation to Th17 cells. The increased number of pathogenic Th-17 cells might participate in the initiation and maintenance of SLE. Th-17 cell is a kind of CD4⁺T cell that promotes occurrence and development of inflammation^[12]. It is generally believed that Th-17 and IL-17 are decisive factors of tissue injury in autoimmune diseases. Autoantigen-specific Th-17 cells are highly pathogenic and can cause severe inflammation and autoimmune diseases^[6,22]. There is a series of evidence showing that Th-17/IL-17 is involved in the pathogenesis of SLE. Abnormal increase of IL-17 level in SLE patients is related with disease activity. The proportion of T cells producing IL-17 in the peripheral blood of SLE patients increases, which are mainly Th17 cells of CD4⁺ and double negative T cells of CD3⁺CD4⁻CD8⁻. Imbalance between Th17 and Treg

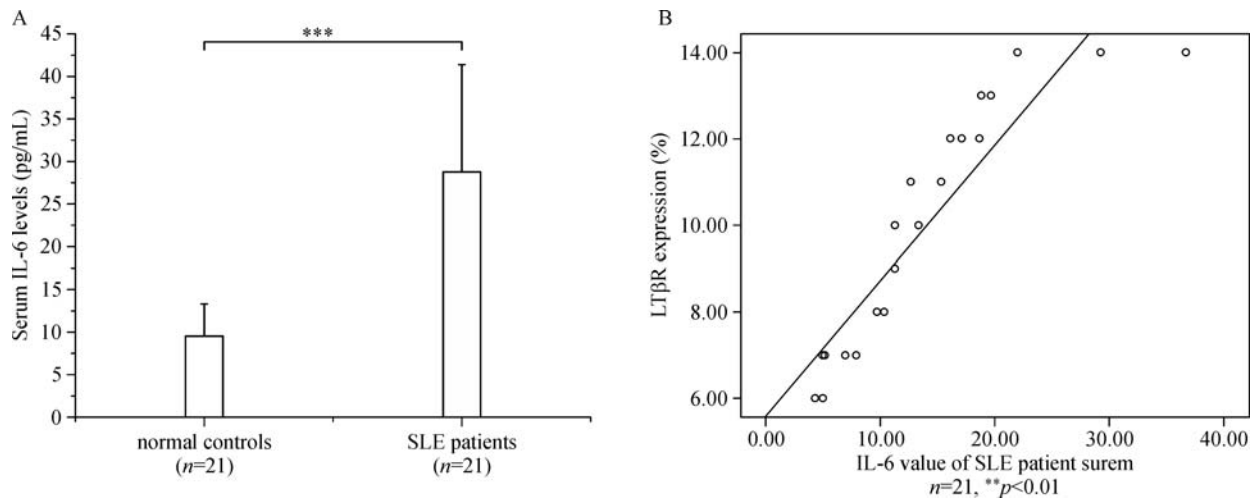


Fig. 9 The high expressions of LT β R in SLE patients are correlated positively with serum levels of IL-6. A: Increased IL-6 level in the serum of SLE patients; B: Expressions of LT β R and IL-6 in SLE patients are positively correlated.*** $P < 0.001$.

cells participate in the pathogenesis of SLE^[11,23–25]. The high expression of IL-17 in SLE can increase pathogenic immune response by promoting inflammation and damage of target organs, for example, IL-17⁺ cells invade into the kidney and participate in lupus nephritis^[11–12]. In recent years, the pathogenic role of Th-17 has become a new hot spot in the study of pathogenesis of SLE, which makes people break through the limitations of understanding of Th1/Th2 imbalance, and study the pathogenesis of SLE in a broader and more targeted perspective^[14–18]. IL-6 is an important factor inducing TH-17 differentiation, while IL-23R signaling has important significance for the maintenance and expansion of Th-17. In 2008, Wong, et al found the level of serum IL-23 and IL-17 increased abnormally in lupus nephritis^[14]. Other studies found that expression of IL-23 receptor in SLE patients T cells increased, and was positively correlated with abnormal increase of IL-17 level in vivo^[18]. The study of lupus model mice also had similar findings and put forward important concept of IL-23/IL-17 axis^[15–17]. The lymphocytes of B6/Ipr rats were isolated, treated with IL-23, and then transferred to the lymphocyte-defect *Rag-1*^{-/-} rats. These rats would develop lupus nephritis, while IL-23R gene defect rat would not^[16]. Here, we demonstrated that in SLE patients, the level of IL-6 was positively correlated with LT β R expression of T cells and LIGHT- LT β R signaling promoted expressions of IL-17A and IL-23R in SLE patients' T cells.

In conclusion, the abnormal high expression of LT β R increased constantly by the positive feedback of LT β R signaling would promote T cell apoptosis and promote IL-23R expression and differentiation and formation of Th-17. These help us to better understand SLE

pathological mechanism through the relationships of LT β R signaling with T cell apoptosis and with Th-17 cell differentiation, and also help us to better understand the therapeutic mechanism of LT β R signal blocking by using LT β R-Ig fusion protein.

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