Original Article

# Increased Macroautophagy in Interferon-Gamma-Producing T Cells from Patients with Newly Diagnosed Systemic Lupus Erythematosus

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## Abstract

**Background:** Imbalance of interferon-gamma (IFN-γ), interleukin (IL)-4, and IL-17 producing by T cells is confirmed to contribute to the pathogenesis of systemic lupus erythematosus (SLE). Autophagy is now emerging as a core player in the development and the function of the immune system. Therefore, we investigated the autophagic behavior in IFN-γ-, IL-4-, and IL-17-producing T cells from patients with SLE.

**Methods:** Thirty patients with SLE and 25 healthy controls matched for gender and age were recruited between September 2016 and May 2017. The autophagic levels in IFN-$\gamma^+$ T cells, IL-$4^+$ T cells, and IL-$17^+$ T cells from patients with newly diagnosed SLE and healthy controls were measured using flow cytometry. The plasma levels of IFN-γ were determined by enzyme-linked immunosorbent assay in SLE patients and healthy controls. Unpaired *t*-tests and the nonparametric Mann-Whitney *U*-test were used to compare data from patients with SLE and controls. Spearman's rank correlation coefficient was applied for calculation of the correlation between parallel variables in single samples.

**Results:** Our results showed increased percentage of autophagy in IFN-$\gamma^+$ T cells from patients with SLE and healthy controls ([8.07 ± 2.72]% vs. [3.76 ± 1.67]%, $t = 5.184$, $P < 0.001$), but not in IL-$4^+$ T cells or IL-$17^+$ T cells ($P > 0.05$) as compared to healthy donors. Moreover, the plasma levels of IFN-γ in SLE patients were significantly higher than those in healthy controls ([68.9 ± 29.1] pg/ml vs. [24.7 ± 17.6] pg/ml, $t = 5.430$, $P < 0.001$). Moreover, in SLE patients, the percentage of autophagy in IFN-$\gamma^+$ T cells was positively correlated with the plasma levels of IFN-γ ($r = 0.344$, $P = 0.046$), as well as the disease activity of patients with SLE ($r = 0.379$, $P = 0.039$).

**Conclusion:** The results indicate that autophagy in IFN-$\gamma^+$ T cells from SLE patients is activated, which might contribute to the persistence of T cells producing IFN-γ, such as Th1 cells, and consequently result in the high plasma levels of IFN-γ, and then enhance the disease activity of SLE.

**Key words:** Autophagy; Lupus Erythematosus; Systemic; T Helper cells

## Introduction

Autophagy is a metabolic process in which cytoplasmic materials are sequestered by the autophagosome and delivered to the lysosome for degradation and recycling.[1,2] Autophagy is now emerging as a core player in the development and the function of the immune system.[3-6] It shapes the immune cell repertoire by interfering with negative and positive selection during lymphocyte development in the thymus, participates

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in host protection by antigen presentation and immune recognition, regulates lymphocyte survival and homeostasis, and mediates cytokine production.[3,4,7-10] Evidences from genetic, cell biology, and animal models suggest that autophagy plays a pivotal role in the pathogenesis of autoimmune diseases, such as rheumatoid arthritis, Crohn's disease, multiple sclerosis, as well as systemic lupus erythematosus (SLE).[11-15]

SLE is an autoimmune disease of unknown etiology characterized by the production of antinuclear autoantibodies and by a broad range of clinical presentations involving almost all organ systems.[16,17] Breakdown of immune tolerance is believed to be one of the major mechanisms which trigger the production of autoantibodies by B cells and antibody-forming cells.[18,19] As such, SLE was thought to be a B-cell-driven disease.[20,21] However, it is difficult for the B cells to become functional antibody-forming cells without the assistance of the helper T cells.[22] The helper T cells are typically grouped for their cytokine production into T helper 1 (Th1)/Th2/Th17, etc. Elevated levels of interferon-gamma (IFN-γ), interleukin (IL)-4, and IL-17 produced by T cells have been documented in SLE.[23-26]

A recent study shows that a deregulation of autophagy has been identified in T lymphocytes from lupus-prone mice as well as in patients with SLE.[27-29] In this study, to elucidate the role of autophagy in T cells producing different cytokines for the development of SLE, we investigated the autophagic behavior in IFN-$\gamma^+$, IL-$4^+$, and IL-$17^+$ T cells.

## Methods

### Ethical approval

All procedures were in accordance with the ethical standards laid down in the 1964 *Declaration of Helsinki* and its later amendments. The Ethics Committee of West China Hospital of Sichuan University approved the study (No. 2016-95). All participants signed written informed consent before inclusion.

### Patients and controls

From September 2016 to May 2017, thirty patients with newly diagnosed SLE (<2 months) were consecutively recruited from four different Chinese rheumatology centers including West China Hospital of Sichuan University, Affiliated Hospital of North Sichuan Medical College, the First People's Hospital of Xinxiang Medical University, and People's Hospital of Xinjiang Uygur Autonomous Region. All patients fulfilled the American College of Rheumatology revised criteria for the classification of SLE.[30] Clinical activity was estimated at the time of blood drawing, according to the SLE Disease Activity Index (SLEDAI).[31]

The study also included 25 age- and gender-matched healthy donors as a normal control group from the same Chinese rheumatology centers above.

### Flow cytometry

Peripheral blood mononuclear cells were isolated by Ficoll-Hypaque density-gradient centrifugation. Then, T cells were isolated using CD3 MicroBeads (Miltenyi Biotec, Bergisch Gladbach, Germany) according to the manufacturer's protocol. As many as $1 \times 10^6$ collected T cells were resuspended in 500 μl of 1 × assay buffer (Cyto-ID® Autophagy Detection Kit, Enzo Life Sciences, ENZ-51031-K200), and incubated for 30 min at 37°C in the dark, followed by washing and resuspension with flow cytometry (FAC) permeabilizing solution (eBioscience, USA) for 10 min at room temperature. The sample tubes were washed twice and incubated with APC- or PE-conjugated IFN-γ-, IL-4-, or IL-17A-specific monoclonal Antibodies (mAb) (eBioscience). Isotype IgG (eBioscience) was used as controls. After washing again, cells were immediately analyzed by flow cytometry. Data were obtained on a FACScan flow cytometer (Becton Dickinson, BD, USA) and the results were analyzed using CellQuest software (Becton Dickinson). Percentage of autophagic cells was calculated by the following formula: frequency (%) = events in left upper quadrant (microtubule-associated protein light chain 3-green fluorescent protein [LC3-GFP]$^+$ − IFN-$\gamma^+$ [or IL-$4^+$ or IL-$17^+$] cells)/total of the events in right and left upper quadrant (total of IFN-$\gamma^+$ [or IL-$4^+$ or IL-$17^+$] cells).

### Enzyme-linked immunosorbent assay

The levels of circulating IFN-γ (0.195–100 ng/ml) in the plasma of patients and controls were determined by commercial specific enzyme-linked immunosorbent assay (ELISA) kits (USCN Life Science and Technology Co., Wuhan, China), according to the manufacturer's instructions. Briefly, standard and analyzed samples were added in duplicate to a 96-well coated microtiter plate and incubated for 2 h at room temperature. After washing, biotinylated polyclonal antibody was pipetted into the wells. Following washing, a substrate solution was added to develop color reaction. Plates were read at 450 nm in an ELISA reader. A curve was prepared by plotting the optical density versus the concentration of the cytokine tested in the standard wells. The concentration of the detected cytokine in the unknown sample was determined by referring the observed optical density to the standard curve.

### Statistical analysis

Variable are expressed as number (percentage) or median (P25, P75). Data were analyzed statistically using unpaired and paired Student's *t*-tests for SLE patients and controls. The nonparametric Mann-Whitney *U*-test was used to compare data from patients with SLE and controls. Spearman's rank correlation coefficient was applied for calculation of the correlation between parallel variables in single samples. The results were analyzed using the 17.0 version of the SPSS statistical software (SPSS Inc., Chicago, IL, USA). A value of $P < 0.05$ was considered statistically significant.

## Results

### Clinical, serological, and immunophenotypic characteristics of patients with systemic lupus erythematosus

The clinical and serological features of the patients studied are summarized in Table 1.

**Table 1: Clinical and serological characteristics of SLE samples**

| Characteristic | Value |
|---|---|
| Age (years) | 30 (15, 58) |
| Male/females | 5/25 |
| Skin rash | 13 (43.3) |
| Photosensitivity | 5 (16.7) |
| Oral ulcer | 10 (33.3) |
| Arthritis | 16 (53.3) |
| Serositis | 8 (26.7) |
| Renal disorder | 10 (33.3) |
| Cytopenia | 18 (60.0) |
| NPSLE | 1 (3.3) |
| ANA | 30 (100.0) |
| Anti-dsDNA | 17 (56.7) |
| Anti-Sm | 8 (26.7) |
| Anti-nucleosomes | 12 (40.0) |
| Glucocorticoids | 17 (56.7) |
| HCQ | 21 (70.0) |
| CTX/MMF | 4 (13.3) |

Data are presented as median (P25, P75) or *n* (%). SLE: Systemic lupus erythematosus; ANA: Anti-nuclear antibodies; NPSLE: Neuropsychiatric lupus; SLEDAI: Systemic Lupus Erythematosus Disease Activity Index; HCQ: Hydroxychloroquine; CTX: Cyclophosphamide; MMF: Mycophenolate mofetil.

### Autophagic activity in interferon-gamma$^+$, interleukin-4$^+$, or interleukin-17$^+$ T cells from patients with systemic lupus erythematosus and healthy donors

To evaluate whether the autophagy was detectable in Th subsets, we examined these cells for the presence of autophagy by flow cytometry. As shown in Figure 1a and 1b, an increased percentage of autophagy was detected in IFN-γ$^+$ T cells from patients with SLE, as compared to healthy donors ([8.07 ± 2.72]% vs. [3.76 ± 1.67]%, $t$ = 5.184, $P$ < 0.001). Whereas, no differences were found in IL-4$^+$ T cells ($P$ = 0.771) or IL-17$^+$ T cells between SLE patients and healthy donors [$P$ = 0.870; Figure 1a and 1b].

### Autophagic activity in relation to cytokines secretion in systemic lupus erythematosus

To evaluate whether the percentage of autophagy is associated with cytokine secretion, the levels of IFN-γ were also determined. The plasma levels of IFN-γ in 30 SLE patients were significantly higher than those in 25 healthy controls ([68.9 ± 29.1] pg/ml vs. [24.7 ± 17.6] pg/ml, $t$ = 5.430, $P$ < 0.001). We found that the percentage of autophagy in IFN-γ$^+$ T cells was positively correlated with the circulating levels of IFN-γ in patients with SLE [$r$ = 0.344, $P$ = 0.046; Figure 2].

### Autophagic activity and clinical profiles of systemic lupus erythematosus

Since increased autophagic activity was found in IFN-γ$^+$ T cells isolated from SLE patients, we further explored whether that altered autophagic activity might influence some clinical features of SLE. An increased autophagy in T cells was defined as the percentage of autophagy greater than the mean of healthy donors (*i.e.*, >8.0%). As shown in Table 2, the incidence of renal involvement was higher in patients with increased percentage of autophagy in IFN-γ$^+$ T cells than that in patients without ($\chi^2$ = 4.286, $P$ = 0.038). Although no statistical differences were found due to relative small numbers of patients, the positive rate of anti-dsDNA and anti-nucleosome antibodies seemed to be increased in patients with increased percentage of autophagy in IFN-γ$^+$ T cells. In addition, we found that the percentage of autophagy in IFN-γ$^+$ T cells was associated with SLE disease activity [$r$ = 0.379, $P$ = 0.039, Figure 3]. Because hydroxychloroquine was demonstrated to be a potent inhibitor of autophagic flux,[32] the percentage of autophagy in IFN-γ$^+$ T cells from 21 patients treated with this drug was compared with 9 patients without this drug, and no significant difference was notated ([8.09 ± 2.88]% vs. [8.01 ± 2.47]%, $t$ = 0.942, $P$ = 0.887).

## Discussion

Autophagy, a lysosome-mediated catabolic process, is responsible for the degradation of long-lived proteins, abnormal aggregates, and damaged organelles, which is essential for maintenance of cellular homeostasis. Hence, autophagy is important for various cellular functions, and its dysregulation is considered to be involved in the occurrence of human diseases including cancer, neurodegenerative disorders, and autoimmune diseases.[33,34]

SLE is a prototypic systemic autoimmune disease characterized by the production of plethora of autoantibodies, formation of immune complexes, and persistent inflammation that can lead to tissue damage.[16,17,35] The production of autoantibodies in SLE is believed to be relevant to multiple malfunctions, including removal of dead cells, scavenging of intracellular DNA and RNA, and control of the long-term survival of B cells and T cells.[35,36] Dysregulation of autophagic processes could be involved in all of these pathologies and may be a core contributor to the development of SLE.[13,37] It has been reported that defect in autophagy results in accumulation of dead cells.[38] Then, excessive dead cell-derived DNA/RNA protein complexes may activate the immune system, leading to the initiation of an autoimmune response.[39] Inappropriate activation and survival of B cells are considered to be critical for sustaining the production of autoantibodies in SLE. In the study by Clarke *et al*.,[40] the modulation role of autophagy in B cell survival was examined in a murine model of SLE. It showed that autophagy-related gene 7 knockout (*Atg7*$^{-/-}$) B cells had reduced survival in culture and failed to efficiently develop into plasma cells. Meanwhile, SLE patients had been reported to have increased B cell autophagic activity. These data suggest that autophagy promotes B cell survival and contributes to their persistence in autoimmune conditions.

T cells play a crucial role in the pathogenesis of SLE in that they enhance the production of autoantibodies by offering substantial help to B cells through stimulating

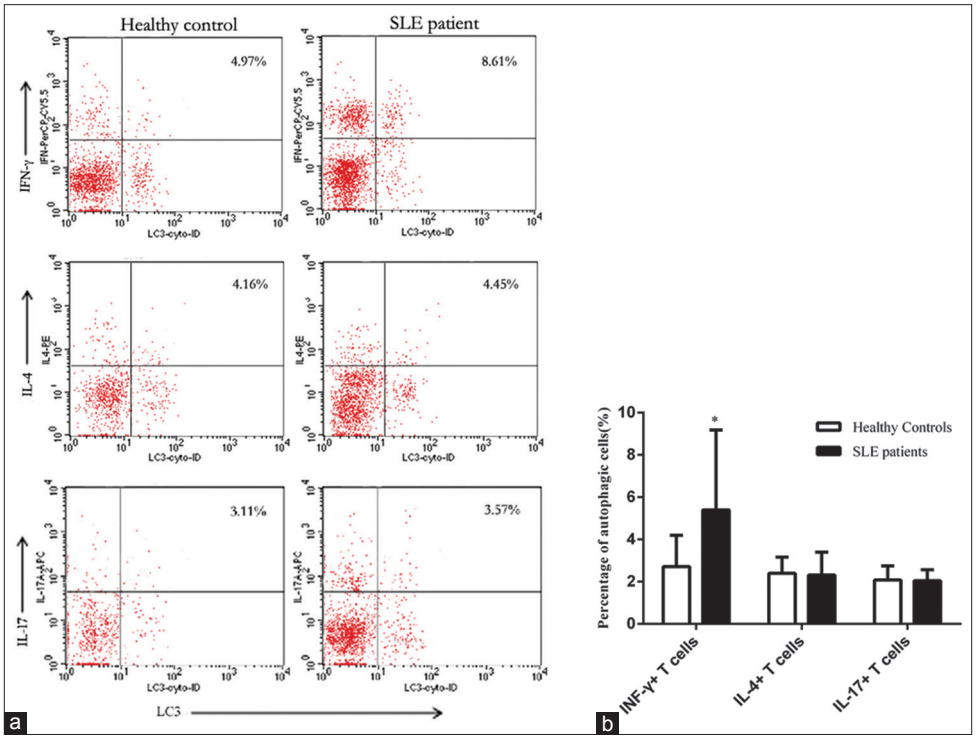


**Figure 1:** Detection of autophagy in IFN-γ$^+$, IL-4$^+$, and IL-17$^+$ T cells from SLE patients and healthy controls. (a) Representative 2-color dot plots from analysis of autophagy and intracellular cytokines by flow cytometry. Numbers in dot plots indicate the percentage of autophagic cells. (b) Summary of autophagy in IFN-γ$^+$, IL-4$^+$, and IL-17$^+$ T cells. Data are presented as means ± standard deviation of independent experiments performed in T cells from healthy controls ($n = 25$) and from SLE patients ($n = 30$). *SLE patients versus healthy controls, $t = 5.184$, $P < 0.001$. LC3: Microtubule-associated protein light chain 3; IFN: Interferon; IL-4: Interleukin-4; SLE: Systemic lupus erythematosus.

**Table 2: Comparison of clinical features between SLE patients with or without increased autophagy in IFN-γ$^+$ T cells**

| Parameters | Increased percentage of autophagy in IFN$^+$ T cells | | $\chi^2$ | *P* |
|---|---|---|---|---|
| | Yes ($n = 16$) | No ($n = 14$) | | |
| Skin rash | 8 (50.0) | 5 (35.7) | 0.621 | 0.431 |
| Photosensitivity | 3 (18.8) | 2 (14.3) | 0.107 | 0.743 |
| Oral ulcer | 6 (37.5) | 4 (28.6) | 0.268 | 0.605 |
| Arthritis | 10 (62.5) | 6 (42.9) | 1.158 | 0.282 |
| Serositis | 4 (25.0) | 4 (28.6) | 0.049 | 0.825 |
| Renal disorder | 8 (50.0) | 2 (14.3) | 4.286 | 0.038 |
| Cytopenia | 11 (68.8) | 7 (50.0) | 0.324 | 0.569 |
| NPSLE | 1 (6.3) | 0 (0.0) | 0.905 | 0.341 |
| ANA | 16 (100.0) | 14 (100.0) | | NS |
| Anti-dsDNA | 11 (68.8) | 6 (42.9) | 2.039 | 0.153 |
| Anti-Sm | 4 (25.0) | 4 (28.6) | 0.049 | 0.825 |
| Anti-nucleosomes | 9 (56.3) | 3 (21.4) | 3.772 | 0.052 |
| Glucocorticoids | 11 (68.8) | 6 (42.9) | 2.039 | 0.153 |
| HCQ | 12 (75.0) | 9 (64.3) | 0.408 | 0.523 |
| CTX/MMF | 3 (18.8) | 1 (7.1) | 0.871 | 0.351 |

Data are presented as *n* (%). SLE: Systemic lupus erythematosus; ANA: Anti-nuclear antibodies; IFN-γ$^+$: Interferon-gamma$^+$; NPSLE: Neuropsychiatric lupus; HCQ: Hydroxychloroquine; CTX: Cyclophosphamide; MMF: Mycophenolate mofetil; NS: No significance.

the latter to differentiate, proliferate, and mature.[22,41] A number of studies have demonstrated that autophagy plays a fundamental role at various stages of T cell differentiation and maturation and is essential for the biology of T cells. Autophagy regulates the T cells selection and the generation of T cell repertoire by presenting intracellular antigens to Major Histocompatibility Complex (MHC) class molecules, controls T cells survival and homeostasis, and participates in the activation and subsequent function of T cells.[42] Gros *et al*.[27] explored autophagic activity by analyzing LC3 conversion as an indicator in T cells from two distinct lupus-prone mouse models and human lupus patients. They found that autophagic activity was deregulated in peripheral T cells from both lupus-prone mice and SLE patients.

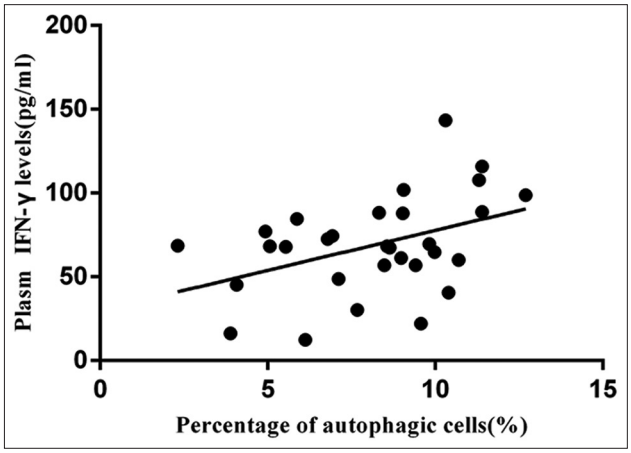


**Figure 2:** Correlation between circulating IFN-γ levels and percentage of autophagy in IFN-γ$^+$ T cells from SLE patients ($r = 0.344$, $P = 0.046$). IFN-γ: Interferon-gamma; SLE: Systemic lupus erythematosus.

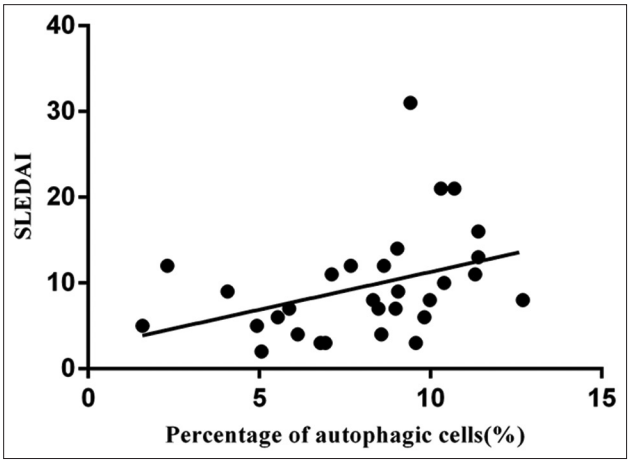


**Figure 3:** Correlation between the SLEDAI score and the percentage of autophagic cells in SLE patients ($r = 0.379$, $P = 0.039$). SLE: Systemic lupus erythematosus; SLEDAI: Systemic Lupus Erythematosus Disease Activity Index.

Alessandri *et al*.[43] also demonstrated that CD4$^+$-naive T cells from SLE patients had an increased level of autophagy than that from healthy donors.

In this study, we addressed the autophagic behavior in T cell subsets from patients with SLE and from healthy donors. We documented an increased autophagic activity in IFN-γ$^+$ T cells from patients with SLE. Whereas, no significant differences in the level of autophagy were found in IL-4$^+$ T cells or IL-17$^+$ T cells between patients with SLE and healthy donors.

IFN-γ, one of the Th1 cytokines, plays a principal role in the development of SLE. Elevated levels of IFN-γ in SLE patients were abundantly reported.[44] Increased levels of IFN-γ were also determined in our patients and were significantly correlated to the autophagic activity in IFN-γ$^+$ T cells from SLE patients. The findings suggested that autophagy might promote the survival of T cells producing IFN-γ and consequently contribute to the increased production of IFN-γ in SLE.

It has been reported that autophagic activity in B cells from SLE patients was positively correlated with the SLEDAI score.[40] We also found that the percentage of autophagy in IFN-γ$^+$ T cells was associated with SLE disease activity ($r = 0.379$, $P = 0.039$). Moreover, we found that patients with lupus nephritis had higher percentage of autophagy in IFN-γ$^+$ T cells as compared with that in patients without lupus nephritis (9.90% [9.02%, 11.33%] vs. 5.83% [4.03%, 8.74%], $Z = 2.640$, $P = 0.008$).

There are some limitations of the study: since CD3$^+$ cells include CD4$^+$ and CD8$^+$ T cells which can both produce IFN-γ, further study focusing on CD4$^+$ T cells is needed to clarify whether autophagy is dysregulated in Th subsets. Furthermore, to recruit more treatment-naïve patients in future study will exclude the impact of therapeutics on autophagic activity. Moreover, the relationship of autophagic activity with clinical features, especially with lupus nephritis, is needed to be further proven in a large number of patients.

In conclusion, we documented an increased autophagic activity in IFN-γ$^+$ T cells from patients with SLE, which was correlated with the production of IFN-γ. The findings indicated that deregulation of autophagy in IFN-γ$^+$ T cells might contribute to the pathogenesis of SLE possibly via prolonging the survival of Th1 and promoting proinflammatory cytokine production. Although the precise mechanisms leading to increased autophagic activity in SLE are still not understood, our and others data suggest that restoring autophagic activity might be an important therapeutic goal in SLE.


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## Conflicts of interest

There are no conflicts of interest.

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# 新发系统性红斑狼疮患者分泌干扰素-γ的T细胞自噬水平增高


## 摘要

**背景：**已证实T细胞产生的干扰素(IFN)-γ,白细胞介素(IL)-4和IL-17比例失衡在系统性红斑狼疮（systemic lupus erythematosus，SLE）发病过程中发挥重要作用。自噬在促进免疫系统发育及维持免疫系统功能方面至关重要。因此，我们探讨SLE患者外周血分泌IFN-γ、 IL-4 或IL-17 的T细胞中自噬发生情况。

**方法：**从2016年9月至2017年5月招募30例新诊断的SLE患者及与其性别年龄匹配的25例健康对照者。通过流式细胞术检测SLE组和健康对照组外周血IFN-$\gamma^+$细胞、IL-$4^+$T及IL-$17^+$T细胞自噬水平。通过酶联免疫吸附实验（ELISA）检测SLE组和健康对照组血清IFN-γ 水平。未配对*t*检验和非参数Mann-Whitney *U* 检验用于SLE组和健康对照组之间的比较，Spearman检验用于检测变量之间的相关性。

**结果：**结果显示SLE患者IFN-$\gamma^+$ T细胞自噬活性较健康对照组增高([8.07 ± 2.72] % vs [3.76 ± 1.67] %, $t$=5.184, $p$<0.001)，但IL-$4^+$ T细胞或 IL-$17^+$ T细胞自噬发生率在两组均无统计学差异（$P$>0.05）。进一步研究发现，SLE患者血浆IFN-γ水平明显高于健康对照组([68.9±29.1]pg/ml vs [24.7±17.6]pg/ml, $t$=5.430, $p$<0.001)。而且SLE患者IFN-$\gamma^+$ T细胞自噬发生率与血清IFN-γ水平（$r$=0.344，$P$=0.046）和疾病活动度呈正相关($r$=0.379, $p$=0.039)。

**结论：**SLE患者IFN-$\gamma^+$ T细胞自噬活性增高，这可能有助于产生IFN-γ T细胞如Th1细胞持续存在，并导致血清IFN-γ水平增高，进而促使SLE患者病情活跃。