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FGL1-LAG3 axis impairs IL-10-Producing regulatory T cells associated with Systemic lupus erythematosus disease activity

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ABSTRACT

Background: Systemic Lupus Erythematosus (SLE) is a prototypic autoimmune disease, which is accompanied by liver damage. However, it remains unknown whether liver damage is associated with SLE progression.

Method: ology: HepG2 and L-02 cells were stimulated with cytokines, and FGL1 mRNA and protein expression levels were determined using Real-time PCR and ELISA, respectively. Regulatory T cells (T_{reg}) isolated from healthy individuals as well as patients with SLE and SLE and liver damage (SLE-LD) were cultured with autologous effector $CD4^{+}$T cells in the presence of a functional antibody or isotype control. The expression levels of LAG3, CD25, PD-1, CXCR5, ICOS and OX40 were evaluated by flow cytometry. FGL1, IL-10, IL-17a and IL-21 levels in serum or culture supernatants were quantified by ELISA.

Results: Patients with SLE-LD exhibits higher disease activity indices and anti-dsDNA antibody levels. Importantly, fibrinogen-like protein 1 (FGL1), a key factor released from the injured liver, is up-regulated in patients with SLE-LD and is associated with disease activity. FGL1 expression is induced by the inflammatory cytokine IL-6 signaling in hepatocytes. Higher expression of the FGL1 receptor lymphocyte activation gene 3 (LAG3) is detected in T_{reg} cells from patients with SLE-LD. The FGL1-LAG3 signaling axis inhibits T_{reg} cell proliferation and impairs the suppressive

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activity of T_{reg} cells by limiting IL-10 secretion. Furthermore, FGL1-LAG3 signaling promotes the production of pathogenic IL-17a and IL-21 by $CD4^+$T cells by reducing IL-10 level produced by T_{reg} in patients with SLE.
Conclusions: The FGL1-LAG3 signal axis is a key mechanism that subverts the suppressive function of T_{reg} cells. This may provide a new therapeutic target for SLE and SLE-induced liver damage.

1. Introduction

Systemic lupus erythematosus (SLE) is an autoimmune disease characterized by the deposition of immune complexes accompanied by inflammatory and necrotic phenomena in tissues including the kidneys, skin, blood vessels, central nervous system and liver [1]. Although SLE affects multiple organs, the incidence of liver dysfunction has greatly increased. The reported incidence of liver dysfunction in patients with SLE ranges from 8.7 to 60 % [2,3]. SLE-associated immune factors include ethanol intake, drug toxicity, viral infection and overlapping autoimmune liver diseases causing liver damage [3]. Liver dysfunction in patients with SLE is usually mild and is considered as an accompanying symptoms of SLE development and treatment [2]. However, liver damage makes SLE treatment much more difficult [2]. Although researchers have observed that liver injury is correlated with the biomarkers of autoimmunity and disease activity in SLE patients [4,5], the correlation between liver dysfunction and SLE activity remains unknown.

In SLE, immune abnormalities are characterized by autoantibody synthesis, B cell hyperactivity, increased lymphoid cell apoptosis, and an imbalance of T-helper-cell (Th) polarization [6]. As one of the contributors to SLE, the Th17 cell is characterized by the production of interleukin-17a (IL-17a) and is associated with disease activity in SLE [7]. During the progressive phase of SLE, Th17 cells infiltrate the kidney, skin and other organs, and secrete IL-17a to induce local inflammation [8]. Another pathogenic Th subset is the T follicular helper (TFH) cells, which express inducible co-stimulator (ICOS) and OX40 and produce IL-21 to provide crucial support for antigen-specific B cells in the SLE pathogenesis [9–13]. TFH cells abnormally increase in patients with SLE and the inhibition of TFH cells has been proposed for SLE therapy [14–16]. Moreover, the impaired activity of regulatory T cells (T_{reg}) is also suggested to contribute to the pathogenesis of SLE [17,18]. Human T_{reg} express $CD4^+CD25^+$ and transcription factor forkhead box P3 ($Foxp3^+$) ($CD4^+CD25^+FoxP3^+$) phenotype. T_{reg} controls immune responses by the secreting of immunosuppressive cytokines, including transforming growth factor beta (TGF-β) and IL-10 [19,20]. Previous studies report that T_{reg} suppresses the activation and proliferation of effector lymphocytes including Th17 and TFH cells, thereby suppressing the pathogenesis of autoimmune and chronic inflammatory diseases [21–23]. Decreased expression and/or an impaired function of T_{reg} may be involved in the development of autoimmune diseases [17,18]. Therefore, the regulatory mechanism of T_{reg} cells in SLE progression needs further investigation.

Lymphocyte activation gene-3 (LAG3), also known as CD223, has received extensive attention in recent years as a member of the inhibitory receptor family. It is widely expressed in various immune cells including $CD4^+$T cells, $CD8^+$T cells, B cell and NK cells [24]. LAG3 interacts with multiple receptors including major histocompatibility complex (MHC) II, Galectin-3 and fibrinogen-like protein 1 (FGL1) [25]. High LAG3 expression is associated with T cell dysfunction in chronic infections and in the tumor environment [26]. In autoimmune diseases, the downregulation of LAG3 triggers immune tolerance breakdown and hyper-immunity. Studies have shown that LAG3 is the key molecule involved with the maximal suppressive activity of both natural and induced T_{reg} [27]. The ectopic expression of LAG3 on $CD4^+$T cells confers regulatory activity and reduces their proliferative potential [28]. A positive correlation has been observed between the expression of LAG3 and IL-10 mRNA in induced T_{reg} [28], suggesting that LAG3 may be a key regulatory receptor in T_{reg}. In addition, $LAG3^+$ T_{reg} displays lower proliferation and lower IL-10 and TGF-β expression than $LAG3^-$ T_{reg} cells [29]. More importantly, it is also reported that mice with LAG3-deficient T_{reg} cells exhibit alleviated autoimmune diabetes with increased T_{reg} cell proliferation and function [30]. These studies suggest that T_{reg} expressing LAG3 exhibits functional impairment and fail to fully suppress inflammation.

In the present study, we found that FGL1, a liver-secreted protein, is upregulated in patients with SLE and liver damage (SLE-LD) and is correlated with SLE disease activity. The SLE-associated inflammatory cytokine IL-6 induces FGL1 production by hepatocytes. More importantly, FGL1 acts as a soluble ligand of LAG3 and suppresses the immunoregulatory activity of T_{reg}. Furthermore, the FGL1-LAG3 signal axis inhibits IL-10 secretion by T_{reg}, which led to increased polarization of Th17 and TFH cells. In conclusion, we demonstrate a key regulatory role of the FGL-LAG3 signaling axis in T_{reg} cells during SLE, which elucidates the mechanism of SLE progression and may provide a potential target for SLE therapy.

2. Materials and methods

2.1. Ethics statement

The study cohort was approved by the following institutional review boards: Zhongshan City People's Hospital (Zhongshan), Dermatology Hospital of Southern Medical University (Guangzhou), and the Fifth Affiliated Hospital of Sun Yat-sen University (Zhuhai) prior to the commencement of this study (K2018-003).

2.2. Human subjects

This was a preliminary study on the association between the disease activity of SLE and liver damage. The inclusion criteria were

males, and females >18 diagnosed as SLE based on American College of Rheumatology (ACR) diagnostic criteria. Exclusion criteria were patients with malignancy, other autoimmune diseases, and other chronic conditions. Liver damage (LD) was defined as a 2-fold or greater elevation of serum AST or ALT levels above the upper limit of the normal range [5]. 78 patients with SLE and 39 healthy controls (HC) were enrolled in this study. Among the 78 SLE patients, 29 patients had liver damage. They were recruited from June 2019 to November 2019, in Dermatology Hospital, Guangzhou, China. The activity of patients was evaluated using the Systemic Lupus Erythematosus Disease Activity Index (SLEDAI). Clinical data including complement component 3 (C3) and complement component 4 (C4) levels, the antibody titers of anti-double stranded DNA (anti-dsDNA), anti-ribosomal P protein (anti-Rib-P), anti-SSA, anti-SSB and anti-cardiolipin (Acl) were collected from all patients. The clinical characteristics were listed in Table 1, and written informed consents were obtained from all participants.

2.3. PBMCs isolation

Peripheral blood mononuclear cells (PBMCs) were obtained from heparin-anticoagulated fresh blood samples by density gradient centrifugation. In detail, blood was mixed with an equal volume of PBS and added to the upper layer of the lymphocyte separation solution (TBD science, China). The mixture was centrifuged for 20 min at 1800g, and then the intermediate white lymphocyte layer was collected and washed 2 times with PBS. PBMCs were resuspended in PBS and used for the subsequent experiments.

2.4. Cell culture

Human hepatocyte cell lines HepG2 and L-02 were cultured in DMEM culture medium (HyClone, GE Healthcare Life Sciences, USA) supplemented with 10 % FBS (Gibco, Thermo Fisher Scientific, USA), 100 U/ml penicillin (Thermo Fisher Scientific, USA) and 100 U/ml streptomycin (Thermo Fisher Scientific, USA). Human PBMCs and sorted T cells were cultured in 1640 culture medium (HyClone, GE Healthcare Life Sciences, USA) supplemented with 10 % FBS, 100 U/ml penicillin and 100 U/ml streptomycin.

2.5. Flow cytometric analysis

PBMCs were washed twice with PBS containing 1 % fetal bovine serum (FBS) and then incubated with antibodies for 30 min at 4 °C. Cells were then washed again with PBS containing 1 % FBS and fixed with 200 μL 1 % PFA. Samples were examined using an Attune NxT Flow Cytometer (Thermo Fisher, USA). The antibodies used were as follows: anti-Foxp3-Percp cy5.5 (BD, 236A/E7), anti-CD25-PE-cy7(BD, 2A3), anti-LAG3-PE (BD, T47-530), anti-CD4-APC (BD, SK3), anti-CD3-FITC (BD, SK7), anti-OX40-PE-cy7 (Biolegend, Ber-ACT35), anti–ICOS–PE (Biolegend, C398.4A), anti-CD127-PE (Biolegend, A019D5), anti-CXCR5-PE-cy7 (Biolegend, J252D4), anti-PD-1-PerCPcy5.5 (Biolegend, EH12.2H7).

2.6. ELISA

Cells culture supernatants and serum samples were collected for ELISA. Cell culture supernatants were centrifuged at 12,000g for 15 min to remove cell debris. Serum samples were obtained from fresh blood by centrifugation at 2,000g for 10 min. ELISA was carried out in accordance with the manufacturer's instructions. The ELISA kits used were as follows: Human FGL1 ELISA Kit (Biotech, RD-FGL1-Hu-96T), Human Galectin3 ELISA Kit (Biotech, RDR-GAL3-Hu-96T), Human IL-10 ELISA Kit (Biotech, RD-IL10-Hu-96T), Human IL-17a SuperSet ELISA Kit (Genie, HUDC0123-1), Human IL-21 ELISA Kit (Biotech, RD-IL21-Hu-48T).

2.7. Flow cytometric sorting

Cell sorting was performed with a SONY SH800 cell sorter (SONY, Japan). In brief, PBMCs were washed with 2 % FBS in PBS and

Table 1
Comparison of clinical and laboratory characteristics between SLE-LD and SLE patients.

Variables	SLE (n = 49)	SLE-LD (n = 29)	P value
Age (years)	38.41 ± 15.39	39.87 ± 17.25	0.689
Gender (M/F)	42/7	27/2	0.712
SLEDAI-2000, score	13.56 ± 7.51	19.93 ± 10.87	**0.002**
Anti-dsDNA (U/ml)	109.82 ± 72.63	157.09 ± 85.96	**0.008**
Anti-Rib-P, n (%)	7 (14.28)	10 (34.48)	**0.028**
Anti-SSA, n (%)	26 (53)	17 (58.62)	0.653
Anti-SSB, n (%)	10 (21)	8 (27.59)	0.589
Acl, n (%)	3 (7)	4 (13.79)	0.431
b2-GPI, n (%)	4 (9)	5 (17.24)	0.287
C3 (g/L)	0.57 ± 0.31	0.66 ± 0.28	0.190
C4 (g/L)	0.11 ± 0.14	0.14 ± 0.15	0.359

Abbreviation: SLEDAI-2000: Systemic Lupus Erythematosus Disease Activity Index. Data are presented as the means ± standard deviation for age and as the interquartile range for sex. P values were determined by Student's t-test for continuous variables and the Mann-Whitney *U* test for others.

stained with indicated antibodies for 45 min at 4 °C. After staining, cells were washed again and re-suspended in PBS containing 2 % serum for sorting. $CD4^+$T cells ($CD3^+CD4^+$), $CD4^+CD25^-$T cells ($CD3^+CD4^+CD25^-$) and $CD4^+T_{reg}$ ($CD3^+CD4^+CD25^+CD127^+$) were collected in RPMI1640 medium supplemented with 10 % FBS. After sorting, cells were stained and analyzed with CD25 or Foxp3 antibodies to determine the sorting purity.

2.8. In vitro stimulation

For cytokine stimulation, HepG2 cells or L-02 cells were treated with recombinant IL-17a (20 ng/ml), TNF-α (20 ng/ml), IFN-γ (20 ng/ml), IL-6 (20 ng/ml) and TGF-β (10 ng/ml) for 24 h respectively. For drug hepatotoxicity, HepG2 cells were incubated with aspirin (Merck KGaA, Germany) at varied doses for 24 h. For IL-6 blockade, HepG2 cells were treated with anti-IL-6 Ab (5 μg/ml) or control IgG (10 μg/ml) in the presence of 20 % serum from healthy controls or SLE patients for 24 h. For the inhibition of STAT3, HepG2 cells were pretreated with stattic (5 μM, Selleck) or DMSO for 30 min, and then cultured with 20 % serum from SLE-LD patients for 24 h. Purified $CD4^+$ T_{reg} cells were treated with FGL1-Fc fusion protein (10 μg/ml, R&D Systems), Gal3-Fc fusion protein (10 μg/ml, R&D Systems), anti-LAG3 Ab (5 μg/ml, Biolegend, 11C3C65) or control IgG (R&D Systems) in the presence of anti-CD3 Ab (1 μg/ml, BD), anti-CD28 Ab (1 μg/ml, BD) and IL-2 (50 U/ml) for 3 days. The purified $CD4^+$ T cells and $CD4^+CD25^-$ T cells were treated with FGL1-Fc fusion protein (10 μg/ml), anti-LAG3 Ab (5 μg/ml), anti-IL-10R Ab (5 μg/ml, Biolegend, 3F9) or control IgG in the presence of anti-CD3 Ab (1 μg/ml, BD) and anti-CD28 Ab (1 μg/ml, BD) for 3 days. The cultured supernatant was centrifuged at 12,000g for 15 min and collected for the detection of IL-10, IL-17a and IL-21 levels by ELISA.

2.9. Cell proliferation assay

The cell proliferation assay was performed as previously described (9). In brief, sorted T_{reg} cells were incubated with 0.5 μM CFSE at 37 °C for 10 min and washed with RPMI1640 medium supplemented with 10 % FBS. Then cells were incubated with FGL1-Fc or Gal3-Fc proteins, or FGL1-Fc, anti-LAG3, or a combination of both FGL1-Fc and anti-LAG3 for 12 h, after which the cells were activated with recombinant IL-2 (50 U/ml) and anti-CD3 antibody. The proliferation of cells was detected by an Attune NxT Flow Cytometer (Thermo Fisher, America).

2.10. Real-time PCR analysis

The real-time PCR was performed as previously described [31]. Total RNA was isolated using TRIzol reagent (Invitrogen, USA) according to the manufacturer's recommendations. First Strand cDNA synthesis was performed using Synthesis Kit as described previously. For Real-time PCR, the expressions of the genes (human FGL1 and β-actin) were assessed by PCR amplification with Bio-Rad CFX96 real-time detection system using SYBR Green Master Mix kit (Invitrogen, USA). The mRNA level of target gene mRNA relative to β-actin was calculated using the following formula: Relative mRNA expression $= 2^{\text{CT value (−actin −target gene)}}$.

Human FGL1 forward sequence: 5′-GAAGATCAGTCTGGCTGGTGGT-'3; reverse sequence: 5′-TGCCAGGTGTACCAGACAATCC-'3.

Human β-actin forward sequence: 5′-CACCATTGGCAATGAGCGGTTC-'3; reverse sequence: 5′-AGGTCTTTGCGGATGTCCACGT-'3.

2.11. Statistical analysis

All data were analyzed with GraphPad Prism8 software. For three or multiple groups, analysis of variance (ANOVA) was performed. Pearson correlation analysis was used for linear correlation analysis between two variables. Data were shown as mean ± SEM unless otherwise stated (ns, not significant; $*p < 0.05$; $**p < 0.01$; $***p < 0.001$).

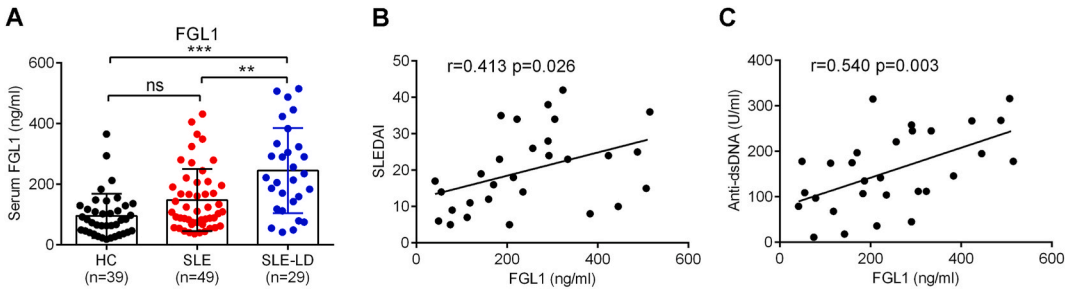

Fig. 1. Increased plasma FGL1 was associated with lupus disease activity in patients with SLE-LD. (A) Serum levels of FGL1 of healthy controls (HC, n = 39), patients with SLE (n = 49) and patients with SLE and liver damage (SLE-LD, n = 29) were measured by ELISA. **(B, C)** The correlations between the serum levels of FGL1 and SLEDAI **(B)**, and anti-dsDNA autoantibodies **(C)** were analyzed by Pearson's correlation test and displayed as scatter plots. One way ANOVA was used in **A**. Pearson correlation analysis was performed in **B** and **C**. Data were shown as mean ± SEM. ns, not significant; $**p < 0.01$; $***p < 0.001$.

3. Results

3.1. Liver damage was associated with lupus disease activity and characterized by increased plasma FGL1

In the cohort, 32.95 % (29/78) of SLE patients met the criteria of liver damage (LD) (Table 1). Among these parameters, age and sex did not influence the prevalence of liver injury in patients with SLE (Table 1). The clinical characteristics and laboratory indices of the study participants are summarized in Table 1. We observed that SLEDAI was higher in SLE-LD patients (19.93 ± 10.87) compared to SLE patients (13.56 ± 7.51; $p = 0.002$; Table 1). Significant differences were observed in anti-dsDNA antibody (Ab) ($p = 0.008$) and anti-Rib-P Ab ($p = 0.028$) levels between the SLE-LD and SLE groups (Table 1). However, the levels of anti-SSA, anti-SSB, Acl, b2-GPI, C3 and C4 Abs in serum samples from the SLE-LD group were not significantly different from those of the SLE group (Table 1). These results indicated that liver damage may be associated with SLE development.

FGL1 is a proliferation and metabolism-related protein secreted by the liver. Its expression can be regulated by tissue-damaging agents or inflammatory stimuli [32]. Here, we found that the concentration of plasma FGL1 was up-regulated in the SLE-LD group

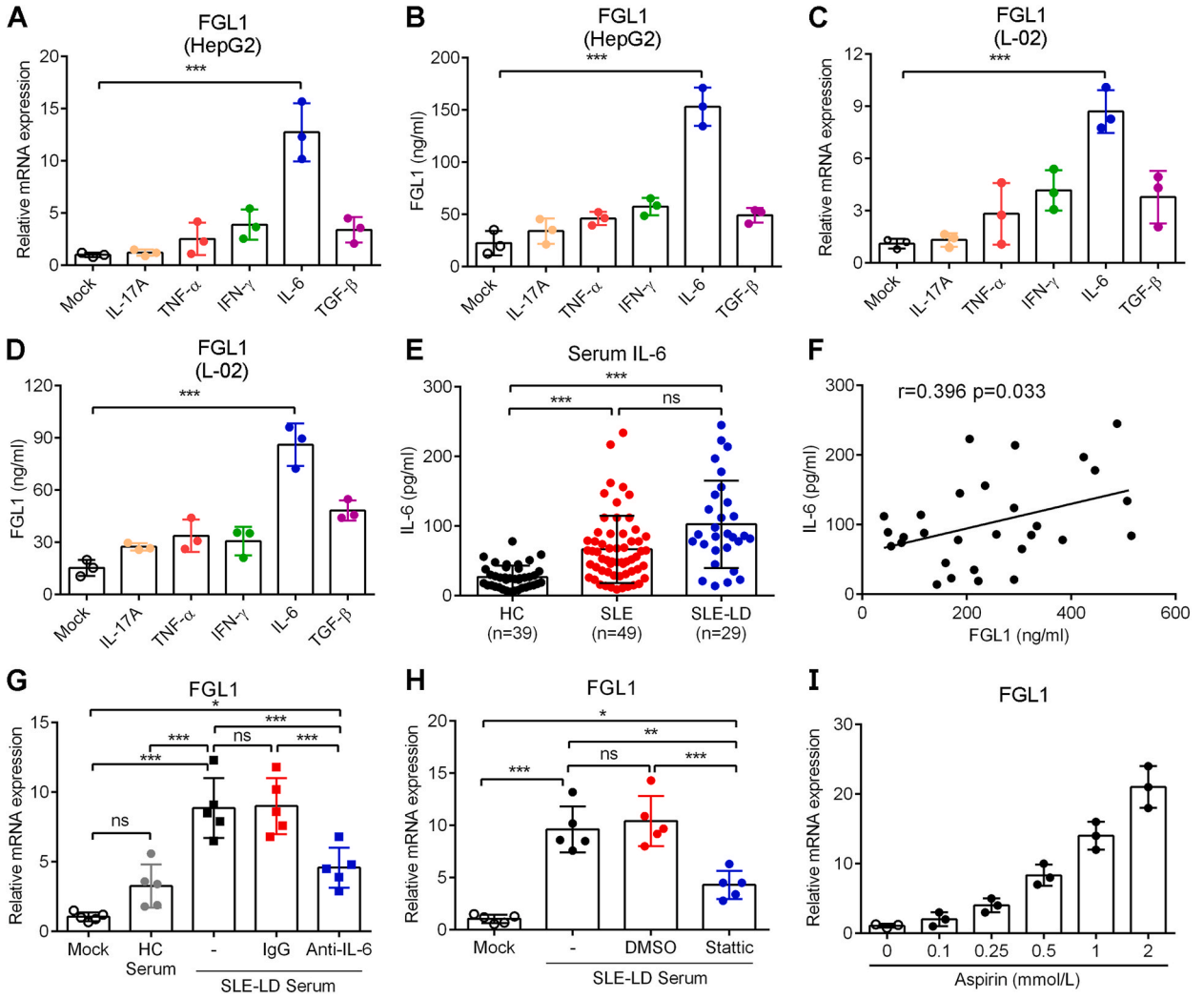

Fig. 2. FGL1 expression was induced by IL-6 signaling in hepatocyte during SLE progression. (A–D) HepG2 (A, B) or L-02 cells (**C, D**) were treated with recombinant IL-17A, TNF-α, IFN-γ, IL-6 and TGF-β respectively. The FGL1 mRNA **(A, C)** and supernatant FGL1 protein **(B, D)** levels were determined by qPCR and ELISA respectively. (**E**) The serum levels of IL-6 of HC (n = 39), SLE (n = 49) and SLE-LD (n = 29) groups were measured by ELISA. (**F**) The correlation between the serum level of IL-6 and FGL1 was analyzed by Pearson's correlation test. **(G)** HepG2 cells were treated with 20 % serum of HC or SLE-LD patients (n = 5) in the presence of anti-IL-6 neutralizing Ab or control IgG, and the mRNA level of FGL1 was determined by qPCR. **(H)** HepG2 cells were pretreated with stattic (5 μM) or DMSO, and then stimulated with 20 % serum of SLE-LD patients (n = 5). **(I)** HepG2 cells were treated with aspirin for 24 h at varied doses. The mRNA level of FGL1 was determined by qPCR. One way ANOVA was used in **A-I**, except for **F**, which was analyzed by Pearson correlation test. Data were shown as mean ± SEM. ns, not significant; $*p < 0.05$; $**p < 0.01$; $***p < 0.001$.

compared to the SLE group or healthy controls (Fig. 1A). To investigate whether liver-derived FGL1 is associated with SLE disease activity, we analyzed the correlation between plasma FGL1 and SLE disease biomarkers. The results showed that the concentration of plasma FGL1 was positively correlated with SLEDAI ($r = 0.413$; $p = 0.026$, Fig. 1B) in patients with SLE-LD. Moreover, the concentration of plasma FGL1 was positively correlated with the level of anti-dsDNA antibodies ($r = 0.54$; $p = 0.003$, Fig. 1C) in patients with SLE-LD. Taken together, these results indicated that liver damage characterized by increased plasma FGL1is associated with lupus disease activity.

3.2. FGL1 secretion was induced by the IL-6-STAT3 axis in hepatocyte during SLE progression

Hepatic inflammation induces FGL1 expression, which is mediated by the STAT3 signaling pathway [33]. To analyze the effect of inflammatory cytokines on FGL1 production, we treated HepG2 cells (human hepatocyte cell line) with recombinant inflammatory cytokines including IL-17a, TNF-α, IFN-γ, IL-6 and TGF-β, which mediate the activation of STAT3 signaling pathway [34–36]. The results showed that the mRNA and protein expression levels of FGL1 were significantly increased upon recombinant IL-6 protein treatment, but remained unchanged upon IL-17a, TNF-α, IFN-γ and TGF-β stimulation (Fig. 2A and B). Similarly, the level of FGL1 was also significantly increased in L-02 cells upon treatment with recombinant IL-6 protein (Fig. 2C and D). Furthermore, serum IL-6 levels in patients with SLE-LD were higher than those in healthy controls, whereas no significant difference was observed between SLE and SLE-LD group (Fig. 2E). Further analysis showed that serum IL-6 levels were positively correlated with serum FGL1 levels in patients with SLE-LD (Fig. 2F). Meanwhile, serum from patients with SLE-LD induced higher FGL1 expression levels than HC-derived serum. Furthermore, blocking IL-6 signaling with an anti-IL-6 neutralizing Ab partly reduced the FGL1 expression induced by SLE-LD serum (Fig. 2G). To further investigate the effect of IL-6/IL-6R-mediated STAT3 signaling pathway on FGL1 expression, we pretreated HepG2 cells with the STAT3 inhibitor Stattic, followed by the treatment with SLE-LD serum. Results showed that inhibition of STAT3 also partly suppressed FGL1 expression in HepG2 cells (Fig. 2H). In addition to SLE related inflammatory cytokines, liver damage can be

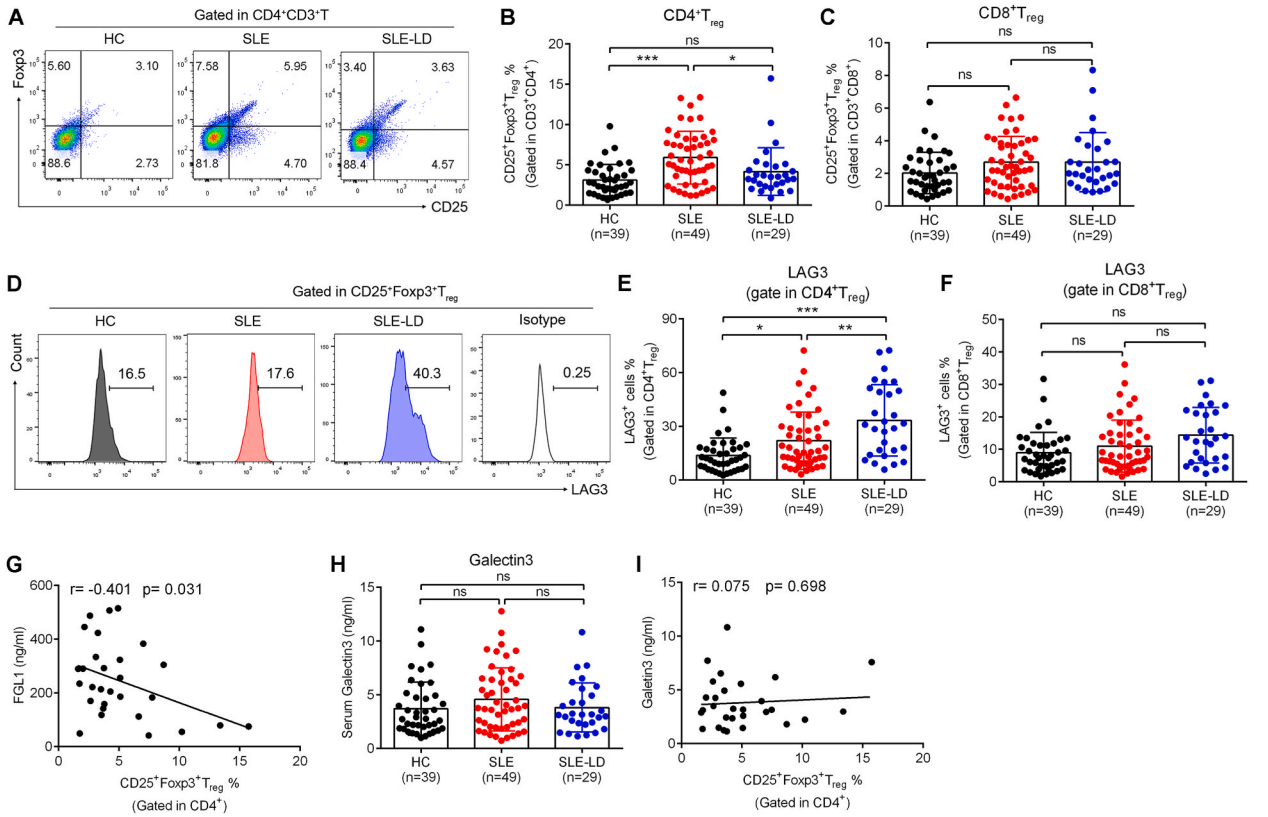

Fig. 3. LAG3 expression was associated with peripheral T_{reg} from patients with SLE-LD. (A–C) PBMCs from HC (n = 39), SLE (n = 49) and SLE-LD (n = 29) groups were analyzed with flow cytometry. $CD4^+T_{reg}$ (**B**) or $CD8^+T_{reg}$ (**C**) were identified as $CD25^+$ $Foxp3^+$ cells within a $CD3^+$ $CD4^+$ or $CD3^+$ $CD8^+$ lymphocyte gate. Representative plots and statistic graph were shown. (**D-F**) Flow cytometric histogram represented the proportion of LAG3 positive cells in $CD25^+FOXP3^+$ subset (**D**). The statistic graph showed the frequency of LAG3 positive cells in each sample (**E, F**). (**G**) The correlation between the serum levels of FGL1 and the frequency of $CD25^+$ $Foxp3^+$ T_{reg} was analyzed by Pearson's correlation test. (**H**) The serum levels of GAL3 of HC (n = 39), SLE (n = 49) and SLE-LD (n = 29) groups were measured by ELISA. (**I**) The correlation between the serum level of GAL3 and the frequency of $CD4^+$ T_{reg} was analyzed by Pearson's correlation test. One way ANOVA was used in **B, C, E, F** and **H**. Pearson correlation analysis was performed in **G** and **I**. Data were shown as mean ± SEM. ns, not significant; *P < 0.05; **P < 0.01; ***P < 0.001.

induced by non-steroidal anti-inflammatory drugs (NSAIDs), which are routinely used to treat SLE patients [37]. To investigate the effect of NSAIDs on FGL1 production, we stimulated HepG2 cells with aspirin. Aspirin increased FGL1 expression in a dose-dependent manner (Fig. 2I), implying that aspirin may regulates immunological homeostasis by increasing FGL1 expression. Collectively, these results suggested that the IL-6/IL-6R-STAT3 signaling pathway promotes FGL1 production in hepatocyte cells.

3.3. SLE-LD resulted in a decreased proportion of peripheral T_{reg} via the FGL1-LAG3 axis

FGL1 has recently emerged as a novel ligand of LAG3 beyond the classic ligand MHC II, which results in the depletion and subsequent dysfunction of T cells, as well as tumor cell escape from immune surveillance [38]. Clinical and experimental studies have observed a T_{reg} imbalance in SLE progression [39]. Here, we detected the proportion of $CD25^{+}Foxp3^{+}$ T_{reg} subsets in the peripheral

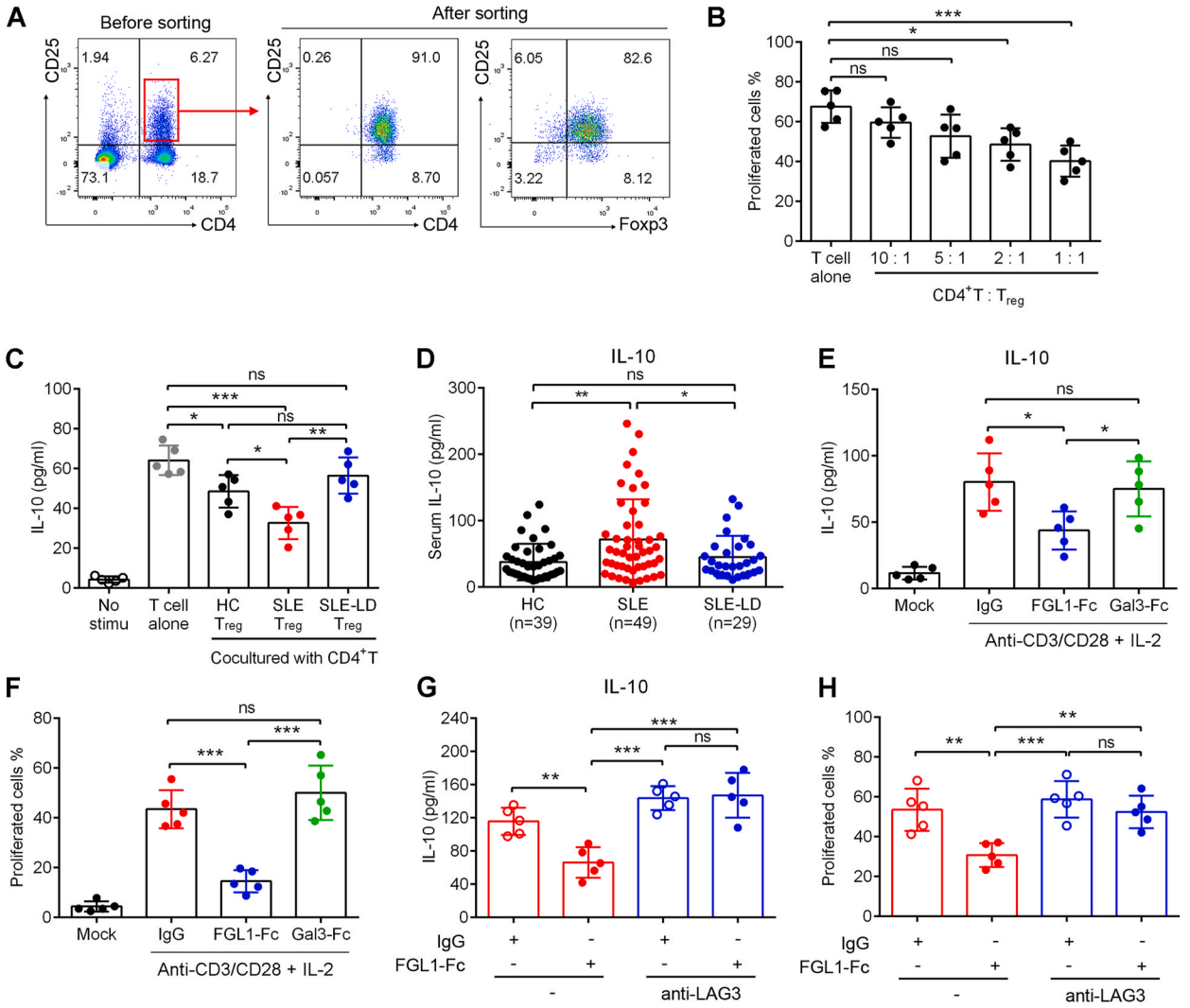

Fig. 4. FGL1-LAG3 signaling axis was associated with the T_{reg} stability in patients with SLE-LD. (A) $CD4^{+}$ T_{reg} (gated in $CD3^{+}CD4^{+}CD25^{+}CD127^{+}$) were sorted from PBMCs by flow cytometry. The cell purity of T_{reg} were determined based on CD25 and Foxp3 staining. **(B)** Sorted $CD4^{+}T_{reg}$ from HC (n = 5) were incubated with autologous $CD25^{-}CD4^{+}T$ cells at indicated ratio in presence of anti-CD3 and anti-CD28 Abs. **(C)** Sorted $CD4^{+}T_{reg}$ from HC, SLE and SLE-LD (n = 5) groups were incubated with autologous $CD25^{-}CD4^{+}T$ cells in the presence of anti-CD3 and anti-CD28 Abs. **(D)** The serum levels of IL-10 of HC (n = 39), SLE (n = 49) and SLE-LD (n = 29) groups were measured by ELISA. **(E, F)** Sorted $CD4^{+}T_{reg}$ from patients with SLE (n = 5) were treated with recombinant FGL1-Fc or GAL3-Fc fusion protein or control IgG in the presence of anti-CD3 Ab, anti-CD28 Ab and recombinant IL-2 protein. IL-10 concentration in cultured supernatant was detected by ELISA and the proliferation of T_{reg} was analyzed by CFSE assay. **(G, H)** Sorted T_{reg} from patients with SLE (n = 5) were treated with anti-LAG3 Ab or control IgG as well as recombinant FGL1-Fc protein. Then cells were treated with anti-CD3 Ab, anti-CD28 Ab and recombinant IL-2 protein. The IL-10 concentration in cultured supernatant was detected by ELISA and the proliferation of T_{reg} or T effector cells were analyzed by CFSE assay. One way ANOVA was performed in B-G. Data were shown as mean ± SEM. ns, not significant; $*p < 0.05$; $**p < 0.01$; $***p < 0.001$.

blood of HC, patients with SLE and patients with SLE-LD. The proportion of $CD4^+T_{reg}$ in SLE and SLE-LD groups was significantly higher than that in control subjects, and the $CD4^+T_{reg}$ frequency in patients with SLE-LD was slightly lower than that in patients with SLE (Fig. 3A and B). However, there was no difference in $CD8^+T_{reg}$ frequency among the three groups (Fig. 3C). These data indicated that liver injury may be associated with reduced $CD4^+T_{reg}$ expression. Next, we determined the LAG3 expression in T_{reg} cells. Flow analysis showed that the expression levels of LAG3 on $CD4^+T_{reg}$ cells were significantly higher in SLE-LD group than in the HC and SLE groups (Fig. 3D and E). However, no significant difference in LAG3 expression was observed in $CD8^+T_{reg}$ among the three groups (Fig. 3F). Furthermore, linear correlation analysis indicated a negative correlation between T_{reg} percentage and FGL1 serum level (Fig. 3G). Since galectin3 (GAL3) is also reported as a soluble ligand of LAG3 [38,40], we examined the serum levels of GAL3 in patients with SLE-LD. No significant differences in GAL3 levels were observed among HC, SLE and SLE-LD groups (Fig. 3H). Further analysis indicated no correlation between serum GAL3 levels and the percentage of T_{reg} (Fig. 3I). These results suggested that the FGL1-LAG3 signaling axis but not the GAL3-LAG3 signaling axis regulates T_{reg} during the development of SLE-LD.

3.4. *FLG1 impaired the immunosuppressive activity of T_{reg} via LAG3 receptor in SLE-LD patients*

T_{reg} cells exert their suppressive effects mainly through cytokines that directly inhibit the activation of effector T lymphocytes [9, 41]. To further explore the role of T_{reg} *in vitro*, peripheral T_{reg} were sorted using flow cytometry. The purity of the sorted cells was 91 % ($CD4^+CD25^+$) and 82.6 % ($CD25^+Foxp3^+$) (Fig. 4A). We firstly conducted a co-culture assay using different ratios of T_{reg} and $CD4^+T$ cells (1:1, 1:2, 1:5 and 1:10). The results showed that HC T_{reg} cells exhibited potent inhibitory effects on $CD4^+T$ cell proliferation at ratios of 1:1 and 1:2, however, no obvious inhibition was observed at ratio of 1:5 (Fig. 4B). We then incubated autologous $CD4^+T$ cells with sorted T_{reg} cells from HC, SLE and SLE-LD patients at a ratio of 1:2, and determined the proliferation of $CD4^+T$ cells after 3 days. We found SLE and HC but not SLE-LD derived T_{reg} cells suppressed the proliferation of $CD4^+T$ cells (Fig. 4C), which indicating that the suppressive activity of T_{reg} was impaired in SLE-LD patients. T_{reg} maintain their suppressive function through their own expansion and the secretion of immunosuppressive cytokines, such as IL-10 [30]. We next measured the serum IL-10 levels in patients with SLE-LD. The results showed that the serum IL-10 levels were higher in patients with SLE than in the HC or SLE-LD groups, and no significant difference was observed between the HC and SLE-LD groups (Fig. 4D). Subsequently, we investigated the effects of LAG3 on the secretion of IL-10 and the proliferation of T_{reg}. Sorted T_{reg} were incubated with recombinant FGL1-Fc or GAL3-Fc proteins, and the

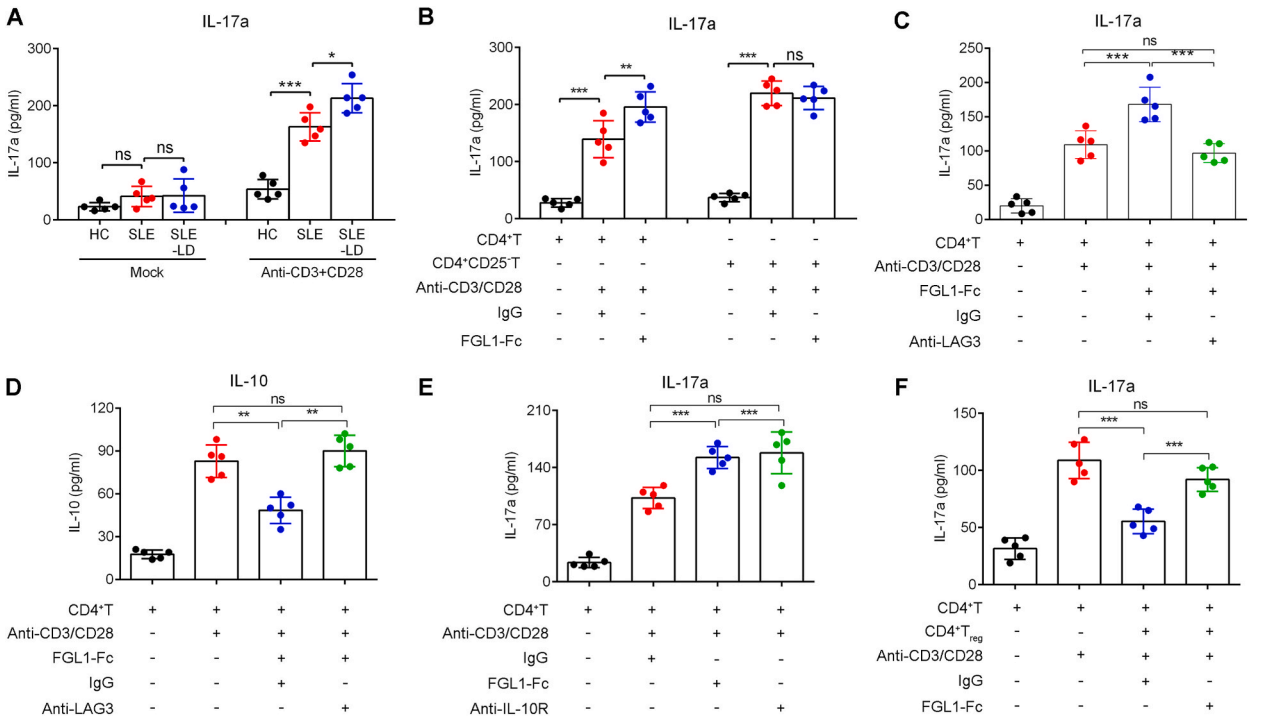

Fig. 5. FGL1-LAG3 signaling axis promoted Th17 polarization through suppressing activity of T_{reg} in patients with SLE-LD. (A) Sorted $CD4^+T$ cells from HC (n = 5), SLE (n = 5) or SLE-LD (n = 5) groups were treated with anti-CD3 and anti-CD28 Abs. **(B)** Sorted $CD4^+$ T or CD4 $^+CD25^-$ T cells (n = 5) were treated with FGL1-Fc fusion protein, after which the cells were stimulated with anti-CD3 and anti-CD28 Abs. **(C, D)** Sorted $CD4^+T$ cells were treated with anti-LAG3 Ab or control IgG in the presence of FGL1-Fc, and then cells were stimulated with anti-CD3 and anti-CD28 Abs. **(E)** Sorted $CD4^+T$ cells were pretreated with FGL1-Fc, anti-IL-10R Ab or control IgG, followed by the stimulation with anti-CD3 and anti-CD28 Abs. (**F**) Sorted $CD4^+T_{reg}$ were incubated with autologous $CD25^-CD4^+T$ cells at ratio of 1 : 2, and then stimulated with anti-CD3 and anti-CD28 Abs in presence of FGL1-Fc fusion protein or IgG control. The IL-17a or IL-10 concentrations in cultured supernatant were detected by ELISA. Data were shown as mean ± SEM. ns, not significant; $^*p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$.

IL-10 level in the supernatant and the cell proliferation were measured to assess the response of T_{reg} cells to anti-CD3 Ab, anti-CD28 Ab and IL-2 stimulation. We observed that the FGL1-Fc protein suppressed IL-10 production and the proliferation of T_{reg} cells, whereas the GAL3-Fc protein had no significant effect on IL-10 secretion and proliferation (Fig. 4E and F). To further demonstrate the role of the FGL-1/LAG3 axis in SLE-LD, T_{reg} were treated with anti-LAG3 blocking Ab and then stimulated with FGL1-Fc protein, anti-CD3 Ab, anti-CD28 Ab and IL-2. The results showed that FGL1-Fc inhibited IL-10 production and the proliferation of T_{reg}, but these effects disappeared when LAG3 was blocked (Fig. 4G and H). Together, these results indicate that the number and function of T_{reg} were significantly disturbed in patients with SLE-LD, and it was the FGL1/LAG3 axis, but not the GAL3/LAG3 signal axis caused the number abnormality or functional dysfunction of T_{reg} in patients with SLE-LD.

3.5. *FGL1-LAG3 signal axis affected Th17 polarization through suppressing the activity of T_{reg} cells in SLE patients*

Th17/T_{reg} imbalance exacerbates SLE development [42], and T_{reg} can suppress Th17 polarization [43]. We sorted $CD4^+$T cells from HC, SLE and SLE-LD groups and stimulated them with anti-CD3 and anti-CD28 Abs to evaluated their differentiation into Th17 cells by detecting IL-17a secretion. The results showed that IL-17a production by SLE and SLE-LD $CD4^+$T cells was higher than that of HC $CD4^+$T cells, and SLE-LD $CD4^+$T cells produced more IL-17a than SLE $CD4^+$T cells (Fig. 5A). To investigate the role of the FGL1-LAG3 signaling axis in the Th17/T_{reg} balance, we treated $CD4^+$T cells or $CD4^+CD25^-$T cells (depleted T_{reg} cells) with FGL1-Fc protein in the

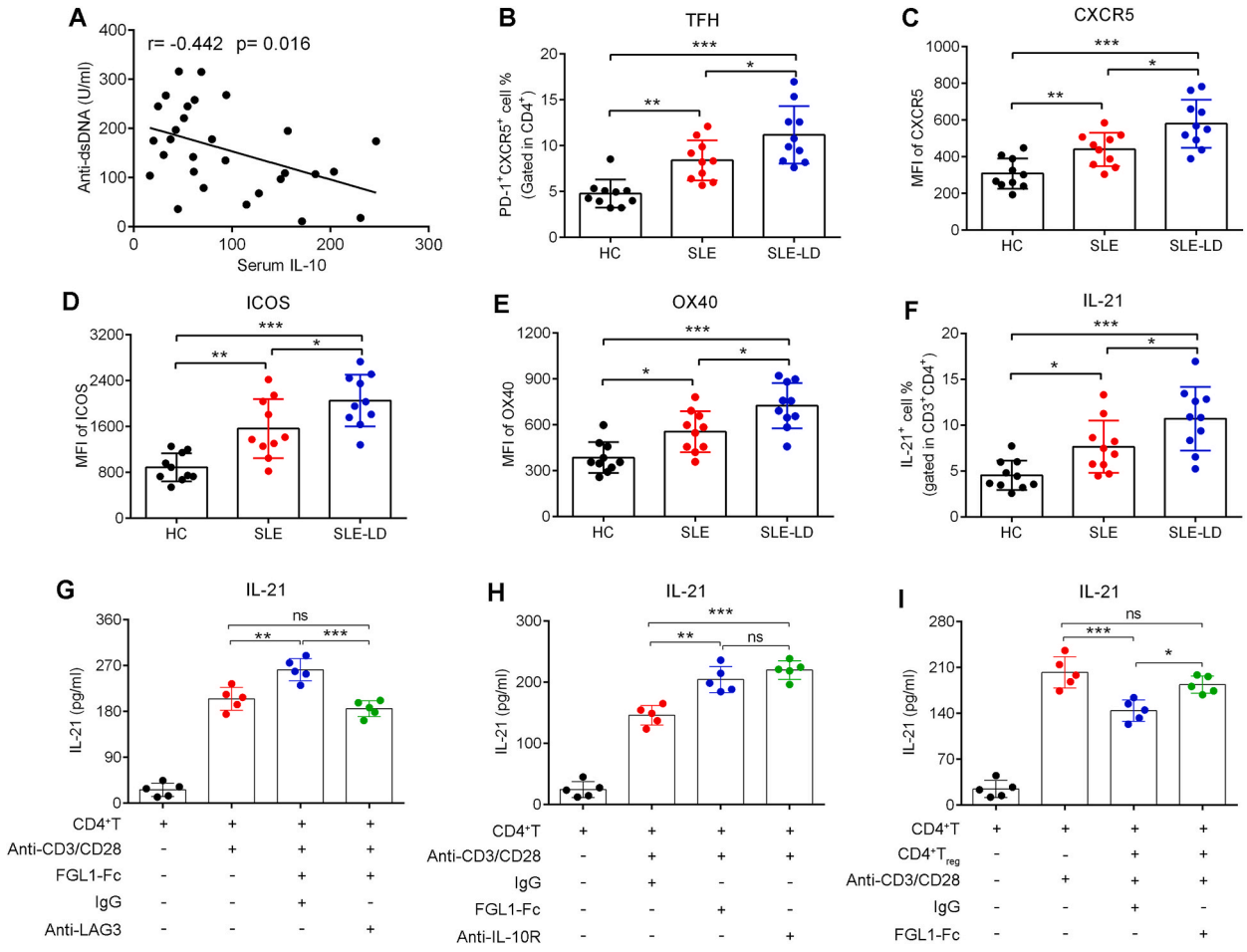

Fig. 6. FGL1-LAG3 signaling axis promoted TFH differentiation in patients with SLE-LD. (A) The correlation between the serum level of IL-10 and anti-dsDNA Ab (n = 29) was analyzed by Pearson's correlation test. (**B–F**) PBMCs were isolated from blood of HC (n = 10), SLE (n = 10) and SLE-LD (n = 10) groups. The expression levels of PD-1^+CXCR5^+ (**B**), CXCR5 (**C**), ICOS (**D**) and OX40 (**E**) (n = 10) were detected by flow cytometry. (**F**) PBMCs from HC (n = 10), SLE (n = 10) and SLE-LD (n = 10) groups were stimulated with PMA and ionomycin, and the intracellular IL-21 was determined by flow cytometry. (**G, H**) Sorted $CD4^+$T cells were treated with anti-LAG3 Ab (**G**), FGL1-Fc, anti-IL-10R Ab (**H**) or control IgG, and then stimulated with anti-CD3 and anti-CD28 Abs. (**I**) Sorted $CD4^+T_{reg}$ were incubated with autologous $CD25^-CD4^+$T cells at ratio of 1 : 2, and then stimulated with anti-CD3 and anti-CD28 Abs in presence of FGL1-Fc fusion protein or IgG control. The IL-21 concentration in cultured supernatant was detected by ELISA. Pearson correlation analysis was performed in **A**. One way ANOVA was used in **B**–**I**. Data were shown as mean ± SEM. ns, not significant; $*p < 0.05$; $**p < 0.01$; $***p < 0.001$.

presence of anti-CD3 and anti-CD28 Abs. FGL1-Fc promoted IL-17a production in $CD4^+$T cells, which contained a T_{reg} subset. However, when the T_{reg} subset was depleted, IL-17a production induced by FGL1-Fc was halted (Fig. 5B). Furthermore, blockade of LAG3 in $CD4^+$ T cells eliminated the enhancement of IL-17a production and the reduction in IL-10 production induced by FGL1-Fc (Fig. 5C and D). These data indicate that the FGL1-LAG3 axis in T_{reg} cells promotes Th17 polarization. To investigate the role of T_{reg} cell derived IL-10 in Th17 differentiation, $CD4^+$ T cells were pretreated with anti-IL10R, and IL-17 level were measured. Results showed that the blockade of IL-10R enhanced IL-17a production (Fig. 5E). Furthermore, to confirm the direct effect of T_{reg} cells on Th17 polarization, we respectively sorted T_{reg} and effector $CD4^+$T cells for co-culture and assessed the production of IL-17a. The results showed that T_{reg} cells directly suppressed IL-17a production of $CD4^+$T cells in the co-culture system, whereas activation of FGL1 via FGL1-Fc increased IL-17a level through inhibiting T_{reg} cells (Fig. 5F). Taken together, these results indicated that FGL1-LAG3 signal promoted Th17 differentiation, which may be dependent on IL-10.

3.6. *FGL1-LAG3 signal axis promoted TFH differentiation through suppressing T_{reg} cells in SLE-LD patients*

B cell abnormalities are involved in SLE disease progression through the production of autoantibodies such as anti-dsDNA antibodies [6,44]. Here, we found that the anti-dsDNA antibody titer was negatively correlated with serum IL-10 level (Fig. 6A). Since an excessive $CD4^+$TFH cell response is the driver of B cell differentiation and autoantibody production [14], we detected the expression of T cell activation markers ICOS and OX40 as well as TFH-specific markers CXCR5 and PD-1 on $CD4^+$ T cells to assess the activation status of TFH cells. The results showed that the percentage of PD-$1^+$$CXCR5^+$$CD4^+$ TFH cells and expression levels of CXCR5, ICOS and OX40 were upregulated in $CD4^+$ T cells in both the SLE and SLE-LD groups, and patients with SLE-LD had higher expressions than patients with SLE (Fig. 6B–E). Moreover, TFH specific cytokine IL-21 was also significantly increased in $CD4^+$T cells in patients with SLE-LD compared to that in the HC and SLE groups (Fig. 6F). To further investigate the role of the FGL1-LAG3 signaling axis in TFH polarization, we sorted $CD4^+$T cells and treated them with the FGL1-Fc protein and anti-LAG3 Ab alone or in combination. The results showed that FGL1-Fc treatment promoted IL-21 secretion. However, when LAG3 was blocked, the increased IL-21 secretion induced by FGL1-Fc was abrogated (Fig. 6G). In addition, the treatment with the anti-IL-10R Ab counteracted the reduction in IL-21 production (Fig. 6H). Furthermore, to confirm the direct effect of T_{reg} cells on TFH polarization, we respectively sorted T_{reg} and effector $CD4^+$T cells for co-culture and assessed the production of IL-21. The results showed that T_{reg} cells directly suppressed IL-21 production by $CD4^+$T cells in the co-culture system, whereas the activation of FGL1 via FGL1-Fc increased IL-21 level through inhibiting T_{reg} cells (Fig. 6I). In summary, these results indicated that the FGL1/LAG3 axis enhanced TFH polarization by inhibiting IL-10 production.

4. Discussion

Patients with SLE have been largely managed with empiric immunosuppressive therapies, which have substantial organ toxicities and do not always provide adequate control of the disease [45]. Therefore, there is an urgent need to fully understand the pathogenesis of SLE and identify the immune factors involved in the development of efficient therapies for SLE. Here, we found that liver damage could increased disease activity of SLE and elucidated that liver-derived FGL1 suppressed the regulatory function of T_{reg} via LAG3 signaling, thus promoting the differentiation of Th17 and TFH subsets (Fig. 7). Our findings may provide potential strategies for the clinical management of SLE.

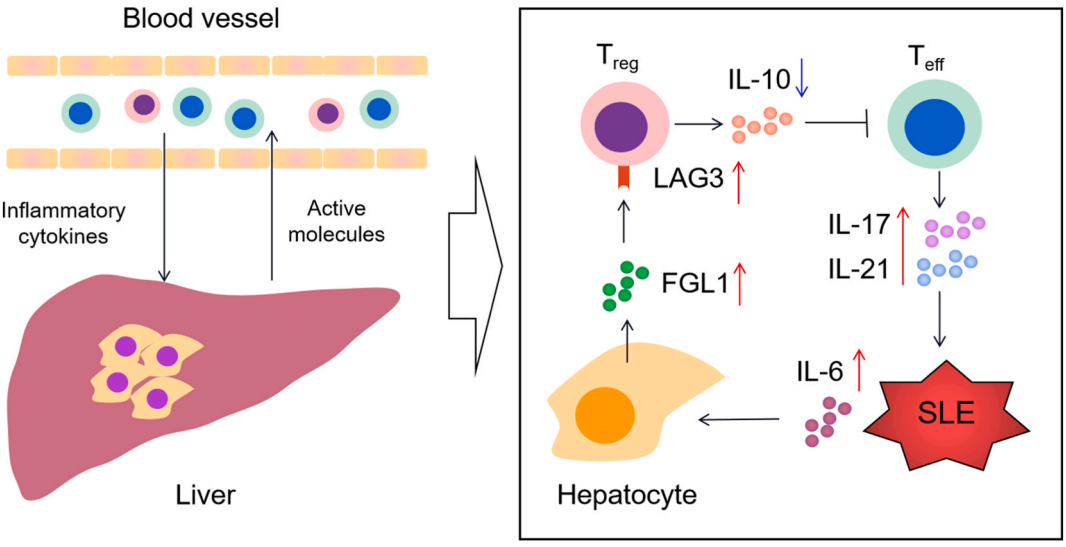

Fig. 7. Graphical abstract. SLE inflammatory cytokine IL-6, induced FGL1 production by hepatocyte. FGL1 interacted with LAG3 to suppress IL-10 secretion by T_{reg}, which increased pathogenic cytokines IL-17 and IL-21 expression by $CD4^+$ T effector cells.

Liver damage in SLE may be caused by: concurrent SLE with other autoimmune liver disease, comorbidity of SLE, nonautoimmune hepatopathy, or lupus hepatitis [2]. Autoimmune hepatitis (AIH), which is triggered by SLE is an overlapping liver injury caused by autoimmune mechanisms [4]. In addition, patients with SLE have comorbidities with many non-autoimmune hepatic diseases such as drug-derived hepatic injury, viral hepatitis, and alcoholic liver diseases [46–48]. SLE induces liver injury via autoantibody production as well as the formation of immune complexes and the inflammatory mediators [45]. For example, hepatic deposition of immunoglobulin G (IgG) is an important pathological factor in the development of liver injury in SLE patients [49]. Contrarily, some researchers have observed that liver injury contributes to disease activity in patients with SLE [4,5], which indicates that there may be a feedback mechanism in patients with SLE with liver damage. Here, we found that SLE caused IL-6 induced FGL1 expression, which may be a pathogenic factor in liver damage during SLE progression. Furthermore, increased FGL1 expression inhibits T_{reg} activity and promotes Th17 and TFH differentiation, further aggravating SLE.

FGL1 is a physiological factor secreted by the liver and is a product of hepatocyte regeneration. Its expression can be regulated by exogenous injuries or stimuli [32]. It is involved in the regulation of multiple functions, including liver metabolism, blood circulation, and lipid metabolism [32,50,51]. FGL1 has been identified as a potential biomarker and plays critical roles in metabolic diseases [52], autoimmune diseases [53] and infectious diseases [54]. Moreover, as an emerging immune checkpoint, FGL1 has been reported to affect cancer development and tumor escape [32]. Plasma FGL1 expression is increased in radiation-induced human hepatocytes [55]. In addition, inflammatory cytokines such as IL-6 and TGF-β can also affect the expression of FGL1 [33,56]. Upon IL-6 stimulation, hepatocytes regulate FGL1 transcription via the STAT3 signaling pathway [33]. Moreover, up-regulated FGL1 expression is also observed in hyperlipidemia, insulin resistance and type 2 diabetes patients with non-alcoholic liver disease [32]. Here, we found that FGL1 expression was increased in patients with SLE with liver damage. *In vitro* results indicated that FGL1 was induced in hepatocytes via the IL-6/IL-6R-STAT3 signal pathway.

Abnormal liver function can be caused by multiple factors including SLE-related factors (such as autoantibodies and inflammatory cytokines), viral hepatitis, alcohol toxicity and harmful SLE drugs. Although an association between SLE and liver disease has been observed on multiple occasions, the relationship between liver disease with co-morbidities and drugs has not been well established. Around 80 % of SLE patients are treated with NSAIDs [37]. It is reported that hepatitis, fulminant hepatic failure, cholestasis, and mixed damage can be caused by these compounds [57]. For several years, aspirin has been the most common drug associated with SLE-related liver damage [37]. Here we found that aspirin increased FGL1 expression in a dose-dependent manner, indicating that aspirin may regulate immunological homeostasis by increasing FGL1 expression. A previous study reported that aspirin induces tolerogenic activity in dendritic cells and determines the fate of naive T cells into regulatory phenotype [58]. However, the immunoreactive intensity altered by liver-derived regulators (such as FGL1) is determined by the receptor (such as LAG3) expression level, receptor-expressed cell subset, other signal interferences and biological metabolism, which needs further studies.

As a suppressor of immune cells, the number and the functions of T_{reg} cells are crucial for the maintenance of immune homeostasis in SLE. T_{reg} and SLE are strongly connected. TGF-β induces the generation of T_{reg} which suppresses SLE by maintaining the balance of immune response [59,60]. Dysfunction of T_{reg} cells promotes the development of SLE [61]. Meanwhile, some factors such as gender and sex hormones can affect the susceptibility to SLE by acting on T_{reg} cells [60] or by regulating the expression of interferons and pro-inflammatory cytokines [62]. Moreover, there existed $CD4^+T_{reg}$ and $CD8^+T_{reg}$ play pivotal roles in controlling of SLE. However, the observed number of T_{reg} cells in patients with SLE remains controversial. Several studies find that the number of T_{reg} cells is reduced and their function is limited in SLE [60,63,64]. However, in our study and other studies [65], an increased count of T_{reg} was found in SLE patients compared to that in normal individuals and was positively correlated with the disease activity in SLE patients. Moreover, we found that the number of T_{reg} was lower in patients with SLE-LD than in those with SLE patients. Owing to the structural similarity of CD4 with LAG3, MHC class II has long been considered to be the classical and exclusive ligand of LAG3 for a long time. However, researchers have recently identified a novel soluble ligands of LAG3. It has been found that GAL3, a 31 kDa galactose binding lectin, can interact with LAG3 [38,40]. GAL3 is widely present in different tissues and cell types, and play an important role in the regulation of the immune responses and inflammation [66]. Besides, LAG3 expression is required for GAL3-mediated suppression of $CD8^+$ T cells *in vitro* [40]. Moreover, FGL1 has been identified as a main ligand of LAG3 and is abundantly produced by human tumor cells [38]. The blockade of FGL1-LAG3 signaling provides a new perspective for tumor immunotherapy [38]. In this study, we found that FGL1, but not GAL3, was up-regulated in patients with SLE-LD. Moreover, FGL1 but not GAL3 fusion protein treatment inhibited the proliferation and IL-10 production in T_{reg}. Our data indicate that the FGL1-LAG3 signal axis is a key regulator of T_{reg} function during SLE progression.

Recently, LAG3 has been identified as an immune checkpoint molecule in tumor therapy. $CD8^+$ T cells with highly expressed LAG3 secrete low levels of IL-2, IL-4, IFN-γ and TNF-α, resulting in a state of dysfunction [67,68]. It has been reported that LAG3 is highly expressed in tumor-infiltrating lymphocytes in solid tumors, such as hepatocellular carcinoma [69], gastric cancer, and colorectal cancer [70], and is considered to be a novel inhibitory receptor in clinic in addition to PD-1 and CTLA4 [71]. The role of LAG3 in chronic infections is similar to its role in tumors. For example, LAG3 is co-expressed with PD-1 in $CD8^+$ T cells and mediates T cell exhaustion during lymphocytic choriomeningitis virus (LCMV) infection. LAG3 expression strongly correlates with the severity of LCMV infection [72,73]. In addition, LAG3 expressed in T_{reg} is crucial for T_{reg} function, but the effects of LAG3 appear to vary with disease category. In asthma, LAG3 is a putative marker of T regulatory type 1 cells (Tr1) cells [74,75]. $LAG3^+$ T_{reg} produce high levels of IL-10, which inhibits the activation of effector T lymphocytes in allergic airway inflammation [74,75]. In autoimmune diabetes, LAG3 conditional knockout mice show a low proliferative response of T_{reg} and limited T_{reg} suppressor function [30]. In the current study, we found that the FGL1-LAG3 signal axis suppressed T_{reg} proliferation and downregulated the production of IL-10, which promoted the differentiation of $CD4^+$ T cells into Th17 and TFH cells in SLE.

In summary, our data revealed that the FGL1-LAG3 signal axis inhibits T_{reg} function and promotes pathogenic Th17 and TFH

polarization in patients with SLE-LD. Patients with SLE and liver damage exhibited higher SLEDAI scores and autoimmune antibody titers than patients with SLE alone. The SLE inflammatory cytokine IL-6, induced FGL1 secretion by hepatocytes, and the level of FGL1 positively correlates with SLE disease activity. Moreover, *in vitro* results showed that the addition of FGL1-Fc protein suppressed the response of SLE-LD T_{reg}, which highly expressed LAG3. FGL1-Fc attenuated IL-10 production by T_{reg} by binding to LAG3, which led to an increase in Th17 and TFH immune responses. These observations may provide favorable evidence that the FGL1-LAG3 signaling axis is a potential driver of SLE development.

5. Conclusion

Our study highlights the importance of the LAG3-FGL1 axis in T_{reg} function and pathogenic effector T cell differentiation in patients with SLE-LD. These findings provide insights into the pathogenesis of SLE-LD and offer potential strategies for the clinical translation, which may help develop new therapeutic interventions for SLE with clinical complications, particularly liver damage.

5.1. Study limitations

The small sample size is a limitation of the present study. In this study, we explored the FGL1-LAG3 signaling axis in clinical samples *ex vivo,* but these mechanisms were not investigated in *in vivo* mouse model and require further explorations.

Declarations

Author contribution statement

YW conceived and designed the experiments. YW, XL and SM wrote the paper with the help of other co-authors. KC, XL, YS and YW performed the experiments and analyzed and interpreted the data. MW, DC, SQ, JS, ZW, SM, MZ and JH contributed reagents, materials, analysis tools or data. All authors read the final version of the manuscript and approved the submission.

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Data availability statement

Data included in article/supp. material/referenced in article.

Declaration of interest's statement

The authors declare no conflict of interest.
No additional information is available for this paper.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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