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Reprograming of peripheral Foxp3$^+$ regulatory T cell towards Th17-like cell in patients with active systemic lupus erythematosus

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ABSTRACT

Treg is essential to limit the extend and duration of the immune response, but its stability is still under debate. Here we demonstrate that IL-17-producing Treg cells (Th17-like cells) increased in peripheral blood of patients with Systemic Lupus Erythematosus (SLE). Notably, the Th17-like cells from patient with active SLE were characterized with some phenotype and function of Th17 cells. Upon stimulation, Helios-Foxp3 + CD4+ T cells decrease Foxp3 expression but increase expression of IL-17 and RORγt. Damage associated molecule pattern and inflammatory cytokines are important for induction of IL-17 expression in Treg cells. The Th17-like cells from patients with active SLE lose suppressive function and have robust response to stimulation of autoantigens. We also observed that the level of Th17-like cells in peripheral blood is closely associated with the clinical index of SLE. These findings suggest that instability of Treg plays a critical role in pathogenesis of autoimmune diseases.

1. Introduction

Systemic lupus erythematosus (SLE) is a severe autoimmune disease of unknown origin, which critically breakdowns self-tolerance in its development [1]. Innate and adaptive immune responses against self-antigen induce the production of autoantibodies by B cells. The subsequent deposition of immune complexes in endogenous tissue results in the induction of inflammation [2]. Chronic inflammation caused by auto-immune responses leads to the development of irreversible damage in tissues including kidney. SLE has a variety of clinical manifestations which reflect the multiple and heterogeneous pathways accounting for the expression of disease [3].

Regulatory T cells (Treg) represent a subset of CD4$^+$ T cells that maintain self-tolerance by suppressing autoreactive lymphocytes [4–8].Phenotype and function of Treg cells in SLE have been the focus of intense research for a long time. Contradictory findings regarding quantity and quality of Treg in SLE patients were reported in earlier studies [9], but recent advances in understanding physiology and metabolism of Treg suggested defects of Treg in autoimmune disease [10]. Moreover, new therapeutic strategies involving the improvement of Treg function were shown to improve clinical manifestations in patients with SLE [11,12]. Therefore, a better understanding of molecular events that account for the poor function of Treg in SLE patients will contribute to optimized therapeutic approaches.

Stability of Foxp3$^+$ Treg cell influences the balance between tolerance and autoimmunity, as well as the efficacy of Treg cell-based therapies. Plasticity of Foxp3-expressing Treg cells has been demonstrated to underlie the pathogenesis of food allergy [13] and autoimmune arthritis [14] in mouse models. Notwithstanding results from animal models link instability of Treg cells to autoimmunity, knowledge of these cells in the pathogenesis of human diseases need to be evolved. Recently, a subpopulation of Treg, which expresses IL-17, was found in both healthy donors and patients with autoimmune disease [14,15], but it remains unclear whether this subpopulation represents a transitional form of transformation from Treg to Th17. To demystify this issue, we carried out experiments and confirmed that the expression of IL-17, but

Abbreviations: ANA, anti-nuclear antibodies; CBA, cytometric bead array; CFSE, carboxyfluorescein diaceate succinimidyl ester; CIC, circulating immunocomplex; DAMP, damage associated molecule pattern; DC, dendritic cells; DMSO, dimethyl sulphoxide; FCS, fetal calf serum; GC, germinal center; HD, healthy donor; HMGB-1, High mobility group box 1 protein; IC, immunocomplex; ICOS, inducible costimulator; Ig, immunoglobulin; IL, interleukin; IO, ionomycin; LN, Lupus nephritis; NPLE, Neuropsychiatric lupus; PBMC, peripheral blood mononuclear cells; PC, plasmablasts cell; PD-1, programmed death-1; PMA, 12-O-Tetradecanoylphorbol-13-acetate; RA, rheumatoid arthritis; SLE, systematic lupus erythematosus; SLEDAI, SLE disease activity index; TSDR, Treg-Specific Demethylated Region

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not IL-4 or IFN-γ, increased in peripheral Foxp3$^+$CD4$^+$ T cells in both patients with active SLE and spontaneous lupus model. These IL-17-expressing Treg cells (named Th17-like cells) in active SLE patients show phenotypical and functional characters of conventional T cells. Also, these cells respond to stimulation of autoantigen but show deficient regulatory function. Finally, we confirmed that Th17-like cells can be induced from true Treg cells by HMGB1 and IL-6, which are dramatically increased in active patients, and enhanced stat3 signaling. Collectively, these findings outline a cascade of cellular and molecular events that underlie the conversion of Treg to effector T cells in autoimmune diseases.

2. Materials and methods

2.1. Patients and mice

In this study, a total of 60 patients with SLE who fulfilled 2012 Systemic Lupus International Collaborating Clinics (SLICC) criteria for SLE [16] were recruited. Patients with comorbidities of cancers or infections were excluded. Twenty-eight healthy volunteers served as a healthy control population. The disease activity of SLE patients was assessed using the SLE disease activity index (SLEDAI). Lupus nephritis was diagnosed with renal biopsy. Patients who received any biological or cytotoxic regiment were excluded. Further characteristics of the patients are summarized in Table 1. All participants were given written informed consent prior to their inclusion in the study, which was performed in accordance with the ethical standards laid down Declaration of Helsinki. Ethical approval has been granted by local ethics committee. Mouse strain C57BL/6-Tg(Foxp3-GFP)90Pkraj/J (Foxp3^{GFP+}), MRL/MpJ-Fas^{lpr}/J (MRL-lpr) and control mice MRL-MpJ were all from Jackson Laboratory. Mice were maintained in a specific pathogen-free animal facility and handled in accordance with *Guide for the Care and Use of Laboratory Animals* (Ministry of Health, China, 1998) and with the ethical approval of Shanghai Medical Laboratory Animal Care and Use Committee as well as Ethical Committee of Ruijin Hospital.

Table 1
Characteristics of systemic lupus erythematosus (SLE) patients and control subjects.

	SLE	Control
Number	60	28
Sex (female/male)	60/0	28/0
Age, years, mean ± s.d.	34.50 ± 13.33	35.25 ± 11.61
SLEDAI score, mean ± s.d.	4.98 ± 3.34	n.a
Disease duration, years, mean ± s.d.	6.11 ± 4.32	n.a
C3, mg/dl, mean ± s.d.	73.75 ± 29.26	n.a
C4, mg/dl, mean ± s.d.	15.38 ± 7.20	n.a
IgG, mg/dl, mean ± s.d.	1627.20 ± 681.26	n.a
CRP, mg/ml, mean ± s.d.	4.70 ± 5.74	n.a
ESR, mm/h, mean ± s.d.	34.07 ± 20.66	n.a
Anti-dsDNA, IU/ml, mean ± s.d.	126.2 ± 67.5	n.a
Organ involvement		
Skin involvement, n (%)	24 (40.0%)	n.a
Hematologic involvement (HD), n (%)	25 (41.7%)	n.a
Lupus nephritis (LN), n (%)	30 (50.0%)	n.a
Neuropsychiatric lupus (NPLE), n (%)	6 (10.0%)	n.a
Serositis, n (%)	3 (5.00%)	n.a
Arthritis, n (%)	9 (14.8%)	n.a
Vasculitis, n (%)	3 (5.00%)	n.a
Systemic symptoms (fever, fatigue, etc.), n (%)	33 (55.0%)	n.a
Thrombosis, n (%)	6 (10.0%)	n.a
Immunosuppressive therapy		
Untreated n (%)	7 (11.7%)	n.a
Glucocorticoid (GC) alone, n (%)	8 (13.3%)	n.a
Hydroxychloroquine (HCQ) alone, n (%)	6 (10.0%)	n.a
GC + HCQ, n (%)	46 (76.7%)	n.a
Glucocorticoid dosage, mg/day, mean ± s.d.	9.27 ± 7.65	n.a

Values are in mean ± standard deviation (s.d.); n.a.: not applicable.

2.2. Flow cytometry

Peripheral blood mononuclear cells (PBMC) were isolated from heparinized blood by Lymphocyte Separation Medium (PAA Laboratories) density gradient centrifugation. Five- to eight-color flow cytometry was performed with directly conjugated mAb; dead cells were excluded using Fixable viability stain 510 (BD Bioscience). To analyze cytokine production, cells were stimulated and incubated for 4 h with 50 ng/ml phorbol myristate acetate, 1 μg/ml ionomycin (both from Sigma-Aldrich), and 1 μl/ml GolgiStop (BD Biosciences). After stimulation, cell surface markers were stained with the fluorescently labeled monoclonal antibodies mentioned above. Next, cells were fixed and permeabilised for 60 min at 4 °C in dark with the Foxp3 Fixation/Permeabilisation Concentrate and Diluent buffer set (eBioscience). Then cells were stained with fluorescently labeled mAb against to intracellular antigen. The mAbs for the study include PE/Cy7-IL-17 (BL168), FITC-IFN-γ (4S.B3), PerCP/Cy5.5-IL-4 (MP4-25D2), APC/Cy7-CD4 (RPA-T4), Pacific Blue-Foxp3 (259D), PE-Helios (22F6), APC-RORγt (AFKJS-9), PE/Cy7-CD127 (A019D5), PerCP/Cy5.5-CD25 (BC96), APC-ICOS (C398.4A), PE-CCR6 (G034E3), PerCP/Cy5.5-CTLA-4 (BNI3) and APC-PD-1 (EH12.2H7). Blood cells and tissue infiltrated cells of mice were stimulated and stained as the protocol described above. mAbs include APC-IL-17 (TC11-18H10.1), PerCP/Cy5.5-CD45 (30-F11), APC-RORγt (AFKJS-9), FITC-CD4 (GK1.5) and PE-Foxp3 (MF-14). Cells were examined by flow cytometry (BD CantoII), and data was analyzed using FlowJo software (Tree Star, USA). All the fluorescently labeled mAb and isotype control were purchased from eBioscience and BioLegend. For phosphor-flow analysis of STATs, PBMC were isolated from peripheral blood as described above with a bed-to-bench time not exceeding 30 min. Cells staining was treated as described before [17]. Fluorescently labeled mouse anti-human stat3, stat6 and stat5 were from BD Bioscience.

2.3. Cell culture

Cells were cultured in RPMI 1640 medium (Invitrogen) supplemented with 100 U/mpenicillin/100 μg/ml streptomycin, 0.5 mM sodium pyruvate, 2 mM glutamine, 10 mM HEPES (all from invitrogen), as well as 10% heat-inactivated FCS (Gibico) in 96-well U-bottom plates (Costar) in a humidified CO_2-containing atmosphere at 37 °C. For induction of IL-17 in Treg cells, recombinant protein HMGB-1 (R&D) IL-1β (BD Bioscience) or IL-6 (BD Bioscience) were added into the media, which contained 20 U/ml IL-2 (PerproTech), cells were cultured in αCD3-coated 24-well plates. To check the impact of serum of patients on cells, 20% human AB serum from healthy donors or patients with active SLE was used to substitute for FCS. To detect IL-17 induction in GFP$^+$ Treg cells, isolated Treg cells were stimulated with supernatant of kidney homogenates for 3 days in the presence of recombinant of IL-2. These cells were cultured in αmCD3-coated 24 well plates. Kidney homogenates were acquired according to the protocol described before [18].

2.4. Genomic DNA isolation, bisulfite conversion, and MS-qPCR

Genomic DNA was isolated using DNA isolation kits (ZYMO Research, USA). 0.5–1 μg genomic DNA was bisulfite treated using methylation kits (ZYMO Research, USA) according to the manufacturer's instructions. MS-qPCR was performed using SYBR Green reagent (Thermo Scientific, USA). Primers for methylation-specific Foxp3 were: forward: 5'-CGATAGGGTAGTTAGTTTTCGGAAC-3′, reverse: 5′- CATTAACGTCATAACGACCGAA-3′; and primers for demethylation-specific Foxp3 were: forward: 5′- TAGGGTAGTTAGTTTTTGGAATGA-3′, reverse: 5′- CCATTAACATCATAACAACCAAA-3′. Real-time PCR was

performed in a final reaction volume of 10 μl using the ABI Prism 7900 T Sequence Detection System (Applied Biosystems, USA), containing 25 pmol each of methylation or demethylation-specific forward and reverse primers for Foxp3-TSDR and 25–50 ng of bisulfite-treated genomic DNA template. The methylation rate of Foxp3-TSDR was computed using a formula: $100/[1 + 2^{(CtCG - CtTG)}] \times 100\%$, where Ct_{TG} represents the cycle threshold achieved with TG (demethylated) primers and Ct_{CG} represents the cycle threshold achieved with CG (methylated) primers. For female patients, this rate was corrected by a factor of 2 because one of the two TSDR alleles is methylated as a result of X inactivation.

2.5. Cell separation and suppression assays

PBMCs were purified from fresh heparinized peripheral venous blood using a Ficoll-Hypaque gradient (GE Healthcare, Piscataway, NJ). PBMCs were washed with PBS and resuspended in complete RPMI 1640 media. $CD4^+CD25^{high}CD127^{low}$ Treg cells were purified from PBMC using EasySep™ Human $CD4^+CD127^{low}CD25^+$ Regulatory T Cell Isolation Kit (StemCell Technology) according to manufactory's instruction. The purity of isolated $CD4^+CD25^{high}CD127^{low}$ cells is > 90%. IL-17-producing $CD4^+Foxp3^+$ cells and Th17 cells were sorted on a FACSAria (BD) after pre-enrichment of $CD4^+$ T cells by immunomagnetic negative selection (Stemcell Technologies). $CD4^+GFP^+$ cells ($Foxp3^+$ Treg) were sorted from cells of spleen and lymph node from $Foxp3^{GFP+}$ mice on a FACSAria. To isolate the gland and kidney infiltrated lymphocytes, a previous published protocol [19] was used. For T cell proliferative response, $CD4^+CD25^{high}CD127^{low}$ Treg cells were sorted from PBMC of patients with active SLE and HDs. Purified cells were labeled with carboxyfluorescein diaceate succinimidyl ester (CFSE, Invitrogen) and cultured (1×10^5 per well) in the presence of circulating immunocomplex (CIC) from patients' plasma, 10 ng/ml rIL-2 as well as mitomycin C (Sigma-Aldrich)-treated monocyte-derived dendritic cells (1×10^5 per well) using 96-well U-bottom plates for 3 days. Control cells were stimulated with DC and rIL-2 but without CIC. Suppression assays were performed with 1×10^5 CFSE labeled $CD4^+CD25^-$ T cells and unlabeled IL-17-producing Treg cells at a 2.5:1 ratio in the anti-CD3/anti-CD28 coated 96-well plate. Cells were cultured for 5 days. CFSE dilution was assessed by flow cytometry. Suppression of proliferation was calculated using FlowJo %-divided function.

2.6. Cytokines detection

Concentration of cytokines, including IL-6, IFN-α, IFN-γ, IL-2, IL-4, IL-5, IL-23, IL-12p70, TNF-α and IL-1β were determined by cytometric bead array (CBA, Biolegend) according to manufacturer's instruction. Data was collected on FACS CantoII (BD), and analyzed by CBA software (BioLegend). Concentration of HMGB-1 in serum was determined by HMGB1 ELISA Kit (SHINO-TEST) according to manufacturer's instruction. Absorbance was determined using a Benchmark Microplate reader (BioRad) and data was analyzed using Microplate Manager version 4.0 software (BioRad).

2.7. Preparing autoantigen from serum of patients

Isolation of immune complexes (ICs) and treatment with nucleases and proteases were performed as previously described [20]. Circulating ICs were isolated from patient plasma using the IgG Spin Purification Kit (Pierce), according to manufacturer's instructions. Briefly, SLE plasma was added into the spin columns containing the purification support, and IgG was eluted by centrifuge. Collected ICs were achieved by incubation with immobilized papain (Pierce) at room temperature prior to stimulation of cells.

2.8. Statistical tests

Statistical analysis was performed using Prism 5 for Macintosh (GraphPad) software. Data was shown as mean ± standard error of the mean (SEM). Mann-Whitney and student's *t*-test were applied to compare 2 groups; the 1-way analysis of variance (ANOVA) with Bonferroni's post hoc tests was used to compare 3 or more means. Best fit of correlation was measured by the root mean square. P values < .05 was considered statistically significant.

3. Results

3.1. Increased circulating IL-17-producting $Foxp3^+CD4^+$ Treg in patients with SLE

We carried out multi-color flowcytometry to assay expression of IL-17, as well as IL-4 and IFN-γ, in $CD4^+Foxp3^+$ Treg cells of healthy donors (HD) and patients with SLE (Fig. 1A). As shown in Fig. 1B, the frequency of $IL\text{-}17^+Foxp3^+CD4^+$ (Th17-like cells) cells in total $CD4^+$ was significant higher in patients, especially those with active disease, than that in HDs. However, the frequency of either $IL\text{-}4^+Foxp3^+CD4^+$ (Th2-like cells) or $IFN\text{-}\gamma^+Foxp3^+CD4^+$ (Th1-like cells) was found no difference between HD and patients. Concomitantly, the proportions of $IL\text{-}17^+Foxp3^-CD4^+$ (Th17) cells and $IL\text{-}4^+Foxp3^-CD4^+$ (Th2) cells were much higher in SLE patients with active disease, compared with those of HD. There is no significant difference of the proportion of $IFN\text{-}\gamma^+Foxp3^-CD4^+$ (Th1) between patients and HD (Fig. 1C). Additionally, we found that the frequency of $Cytokines^-Foxp3^+CD4^+$ Treg cells in peripheral blood was much higher in patients than that of HDs (Fig. 1D). Next, we wanted to investigate distribution of Th17-like cells in diseases-involved tissues. Since clinical tissue sample is difficult to obtain, we analyzed the counterparts of human Th17-like cells in spontaneous lupus model, MRL-lpr mouse. Compared with the age and sex-matched congenic strains, much higher levels of $CD4^+Foxp3^+IL\text{-}17^+$ cells were found in peripheral blood of disease mice (supplemental Fig. 1A). Moreover, accumulation of such cells was found in glands and kidney in lupus mice, which were infiltrated with hematopoietic cells (supplemental Fig. 1B). These data suggested that expansion of Th17-like cells is associated with this autoimmune disease.

3.2. Characterization of Th17-like cells in patients with active SLE

We next characterized Th17-like cells both from patients and HDs. As shown in Supplemental Fig. 1C and Fig. 2, Th17-like cells possess characterizes of both Th17 cells and inducible $Foxp3^+$ Treg cells. However, comparing this cell subset between patients and HDs, we found that Th17-like cells isolated from PBMC of SLE patients showed evidence of instable of Treg phenotype, as revealed by downregulation of Foxp3, CD25 and CTLA-4, and upregulation of CD127 (Fig. 2). Data also showed that Th17-like cells from patients with active disease expressed higher level of key canonical markers of Th17, including RORγt and CCR6 (Fig. 2). Additionally, expression of ICOS and PD-1 was markedly increased in those cells of patients with active disease (Fig. 2), consistent with a heightened activation profile [21,22].

3.3. $Helios^-$ Tregs produce IL-17 in inflammatory microenvironment

Phenotypic analysis of Th17-like cells raised question of the origin of these cells in patients. There are two possibilities: $Foxp3^+$ Treg, which is Induced to express IL-17; or Th17, which transiently expresses Foxp3 [23,24]. To classify this question, we stimulated purified $CD4^+CD25^{high}CD127^{low}$ cells and polarized Th17 cells ($CD4^+CD25^-$ cells was cultured in Th17 polarization media and sorted as $CD4^+CCR6^+CD45RA^-$ cells) with pooled serum from patients with severe disease ($n = 5$; all SLEDAI ≥8). Inducible expression of IL-17 was discovered in $CD4^+CD25^+$ cells (Fig. 3A), while little $Foxp3^+$ cells

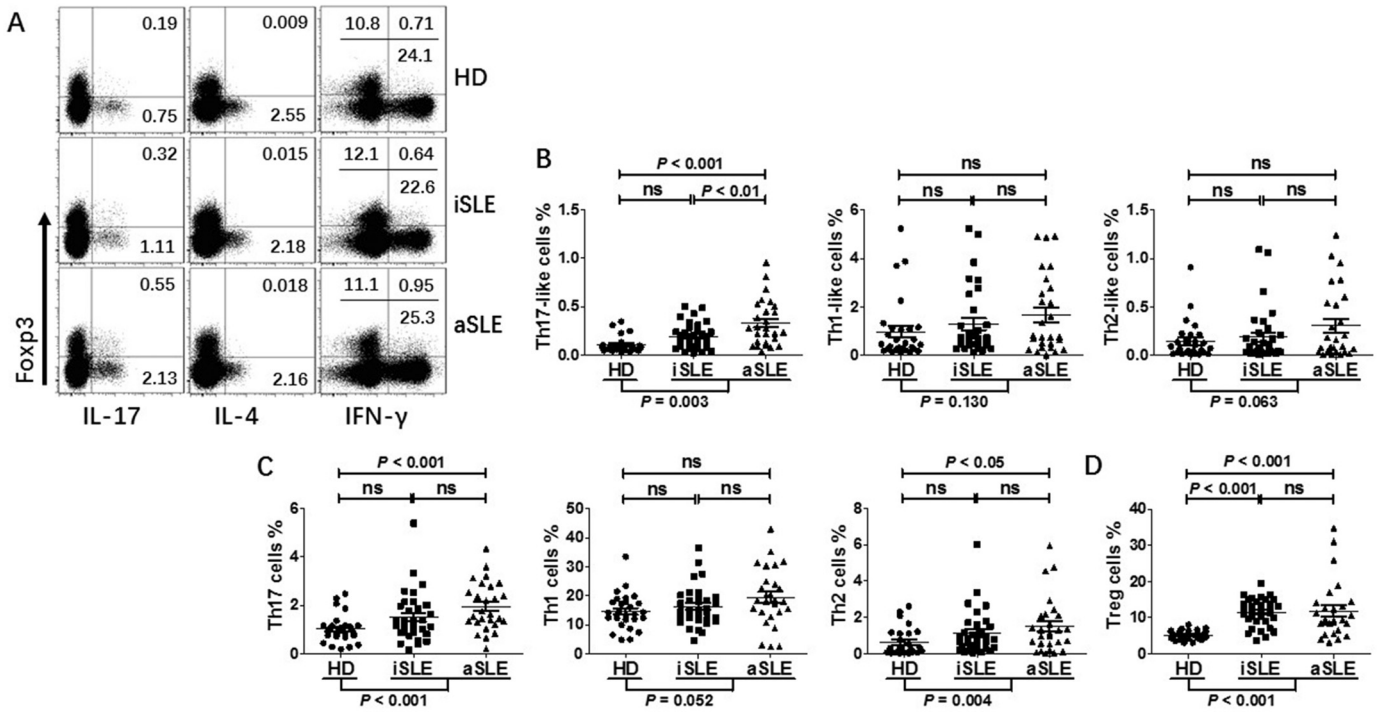

Fig. 1. Frequency of circulating cytokines-expressing Treg in patients with SLE. The expression of IFN-γ, IL-4 and IL-17 in both $CD4^+Foxp3^+$ Treg cells and $CD4^+Foxp3^-$ canonical Th cells from patients with SLE and healthy donors (HDs) was evaluated by intracellular cytokine staining and flow cytometry. (A) Representative plots of IFN-γ, IL-4 and IL-17 expression in circulating $CD4^+Foxp3^+$ Treg cells and $CD4^+Foxp3^-$ Teff cells of healthy donors, patients with inactive SLE (iSLE) and patients with active SLE (aSLE). Numbers in the plots are percentages of gated cells. (B) The percentage of IFN-γ-producing Treg (Th1-like) cells, IL-4-producing Treg (Th2-like) cells and IL-17-producing Treg (Th17-like) cells in total $CD4^+$ T cells was compared among iSLE (n = 31), aSLE (n = 22) and HD (n = 24). (C) The percentage of Th1, Th2 and Th17 cells in total $CD4^+$ T cells was compared among patients with iSLE, patients with aSLEand HD. (D) The percentage of $Foxp3^+$ Treg cells in total $CD4^+$ T cells was compared among iSLE patients, aSLE patients and HDs.

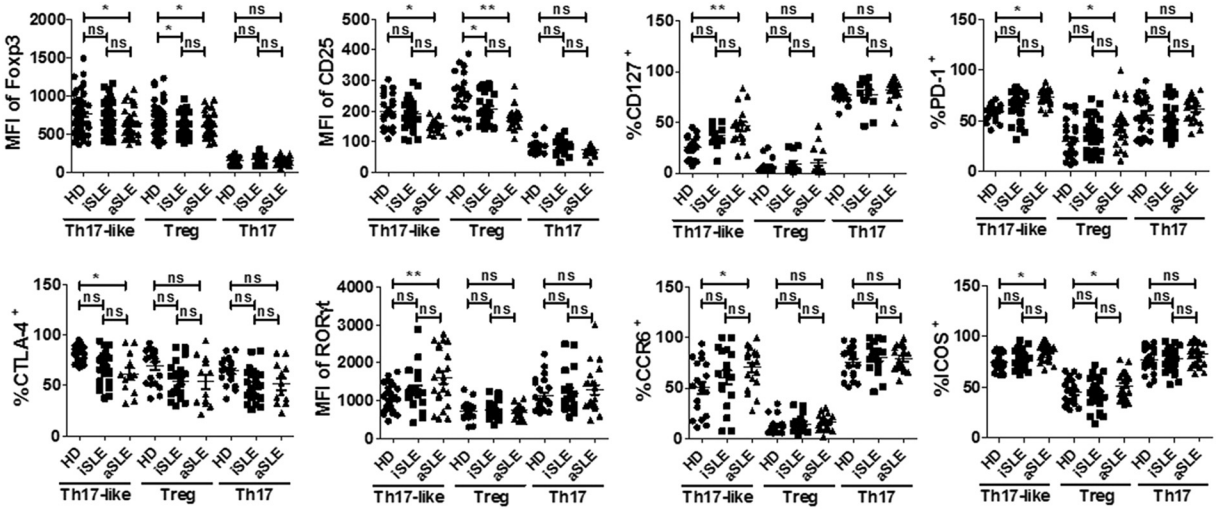

Fig. 2. Characterization of Th17-like cells in PBMC from SLE patients. Surface expression of CD25, CD127, CTLA-4, PD-1, ICOS, and CCR6, and intracellular expression of Foxp3 and RORγt were measured in Treg cells, Th17 cells and Th17-like cells of patients and HDs. The purified PBMCs were examined by flow cytometry after 5 h stimulation with phorbol myristate acetate (PMA)/ionomycin. Expression levels of surface markers or cytokines were quantified as mean fluorescence intensity (MFI; Foxp3, CD25 and RORγt) or positive percent (CD127, CTLA-4, ICOS, PD-1 and CCR6). The expression levels of moleculars measured above were quantified as MFI or percent. *, $P < .05$; **, $P < .01$; ns, not significant.

was observed in Th17 cells (Fig. 3B). To exclude the possibility of contaminated $CD25^+$ Teff cells in $CD4^+CD25^{high}$ cells, which proliferates more robustly than real Treg does, we stimulated GFP^+CD4^+ cells from naïve $Foxp3^{GFP+}$ mice with supernatant of kidney homogenates from MRL-lpr lupus mice in αmCD3-coated plates, and found that these cells dramatically induce expression of IL-17 and RORγt (Supplemental Fig. 2A).

Expression of Helios was once reported to be associated with thymus-derived Treg [22], then we further analyzed the concomitant expression of IL-17 and Helios in cultured Treg. Data showed that $Helios^-Foxp3^+$ Treg cells, rather than $Helios^+Foxp3^+$ Treg cells upregulated expression of IL-17 (Fig. 3C), as well as RORγt. Moreover, $CD4^+Foxp3^+Helios^-$ cells, other than $CD4^+Foxp3^+Helios^+$ cells, significantly decreased expression of Foxp3 upon stimulation of

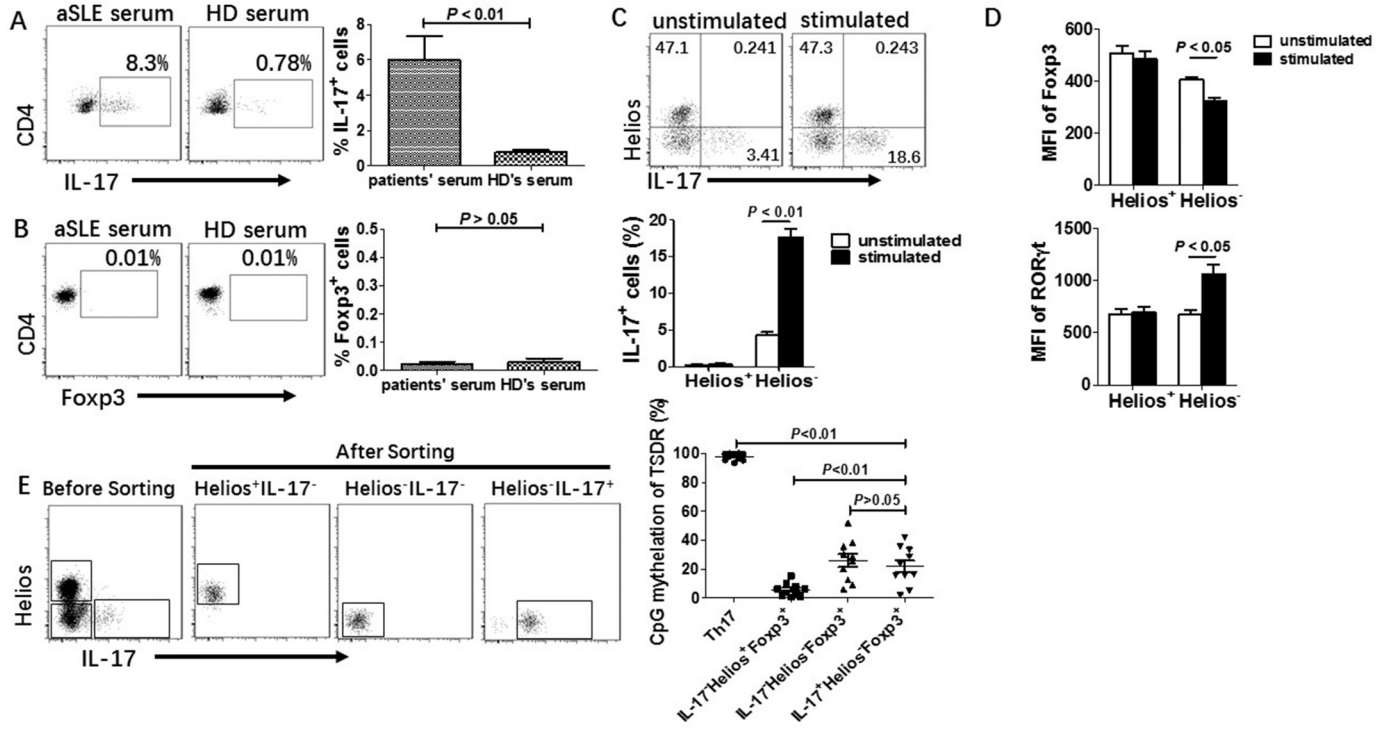

Fig. 3. Helios$^-$ Tregs produce IL-17 in inflammatory microenvironment. (A) Freshly purified CD4$^+$CD25$^+$ cells from HDs were cultured in medium plus pooled serum from active patients (5 patients, all SLEDAI > 8) or HD, and then IL-17 expression were detected by intracellular staining. Typical plots and statistic results were shown. (B) Sorted polarized Th17 cells were cultured as A, and then Foxp3 expression were detected by intracellular staining. Typical plots and statistic results were shown. (C) Freshly purified CD4$^+$CD25$^+$ cells were stimulated by pooled serum from active patients (5 patients, all SLEDAI > 8) or not, and then Foxp3, Helios, RORγt and IL-17 were intracellular stained. Representative data and their quantification (n = 5, right) are shown. (D) The expression level of Foxp3 and RORγt in cells from C was shown. (E) Freshly isolated peripheral blood CD4$^+$CD25$^+$ lymphocytes were stained for intracellular expression of Helios and IL-17. FACS was sorted into three different fractions with purity > 90%: Helios$^+$IL-17$^-$, Helios$^-$IL-17$^-$ and Helios$^-$IL-17$^+$. Left plots show the staining before and after sorting in one representative SLE sample. TSDR methylation was performed in each fraction from 10 HDs and 10 active SLE patients. Median values were shown.

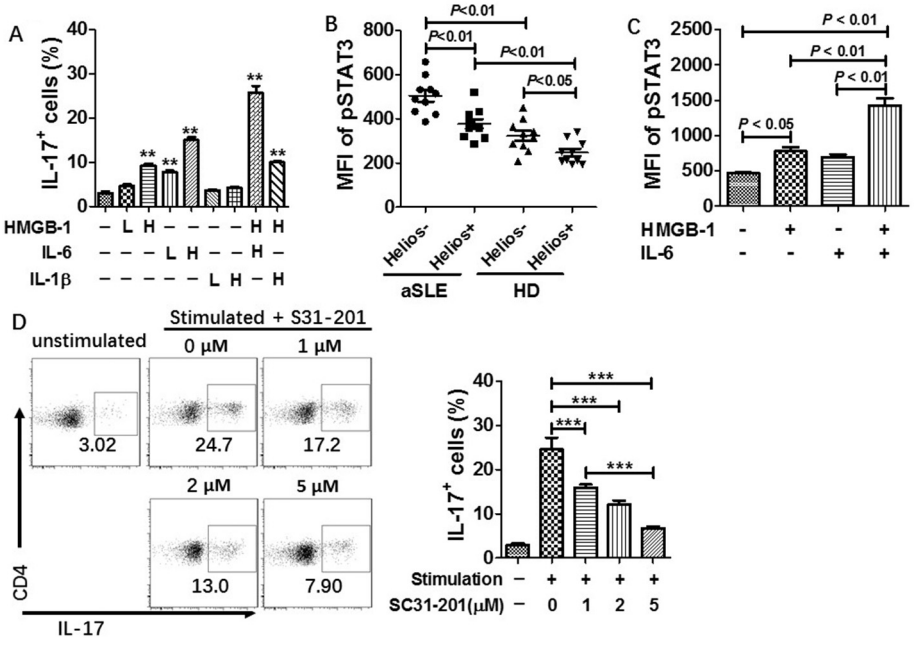

Fig. 4. HMGB-1 and IL-6 induce Th17-like cells through activation of stat3. (A) Freshly isolated CD4$^+$CD25$^+$CD127^{low} cells were stimulated with recombinant proteins HMGB-1 (Low: 5 μg/ml; High: 10 μg/ml), IL-6 (Low: 10 ng/ml; High: 20 ng/ml), IL-1β (Low: 10 ng/ml; High: 20 ng/ml) alone or together as indicated in the presence of IL-2 for 5 days. IL-17 expression was determined by intracellular staining. Data were shown from 4 independent experiments. (B) Quantification of p-Stat3 in Treg cells from healthy individuals (n = 8) and patients with active SLE (n = 8). (C) Quantification of p-Stat3 in Treg cells, which were stimulated by recombinant protein IL-6, HMGB-1 or both. Statistic plots shown the mean and SV from 3 experiments. (D) Inhibition of Stat3 phosphorylation regulates expression of IL-17 in Treg cells. Freshly isolated CD4$^+$CD25$^+$CD127^{low} cells were stimulated with IL-6 plus HMGB-1 in the presence of different concentration of Stat3 inhibitor S31–201 as indicated. Statistic plots shown the mean and SV from 3 experiments.

patients' serum (Supplemental Fig. 2B and Fig. 3D). Methylated status of *Foxp3* locus can be used to distinguish different subsets of Tregs [23,25]. Since Helios expression was reported to be closely associated with methylated status of *Foxp3* locus [17,26], we isolated cells from PBMC, and studied origination of Th17-like cells through epigenetic analysis of methylated status of *Foxp3* Treg-Specific Demethylated Region (TSDR) region. As shown in Fig. 3E, methylated status of Th17-like cells was similar to that of Helios$^-$Foxp3$^+$ Treg cells, but different from Helios$^+$Foxp3$^+$ Treg cells and conventional Th17 cells. Above all, these data strongly suggested that Th17-like cells mainly originate from

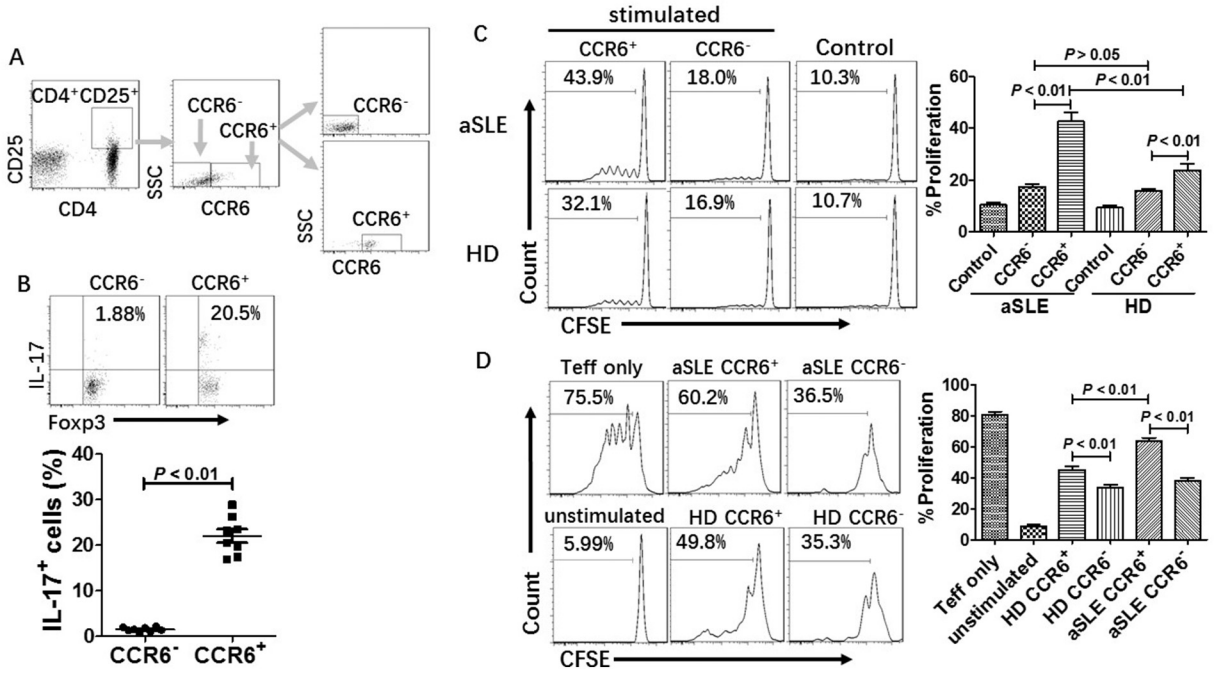

Fig. 5. Deficiency of suppressive function of IL-17-producing Treg cells from patients with active SLE. (A) $CD4^+CD25^+$ Treg cells could be isolated into $CD4^+CD25^+CCR6^+$ subset and $CD4^+CD25^+CCR6^-$ subset. (B) IL-17-producing Treg cells were enriched in the $CD4^+CD25^+CCR6^+$ Treg subset. Representative flowcytometric histogram plot (up) and quantification (down, n = 8) were shown. (C) $CD4^+CD25^+CCR6^+$ cells respond to autoantigen stimulation. Sorted $CD4^+CD25^+CCR6^+$ cells and $CD4^+CD25^+CCR6^-$ cells were co-cultured with $CD14^+$ monocyte-derived dendritic cells, which were pre-treated with purified circulating immunocomplex (CIC), as well as IL-2. CFSE staining for cell proliferation was measure by Flowcytometry 5 days later of co-culturing. Representative histogram plots (left) and quantification (right, n = 5 respectively) were shown. Numbers in the plots indicate the percent of gated cells. Control: cells were stimulated with DC plus IL-2, but without CIC. (D) The $CD4^+CD25^+CCR6^+$ Treg subsets from peripheral blood of active SLE patients showed decreasing suppression activity on $CD4^+CD25^-$ Teff cell proliferation, compared with cells from HD. $CD4^+CD25^-$ Teff cells stained with CFSE were cultured with or without $CD4^+CD25^+CCR6^+$ Treg cells. Teff cells were stimulated with anti-CD3 and anti-CD28 mAb coated beads, and their proliferation in 5 days was determined by flow cytometry. Representative histogram plots (left) and quantification (right) were shown from 5 independent experiments with similar results. Numbers in the plots indicate the percent of gated cells.

canonical $Helios^-$ Treg cells.

3.4. IL-6 and HMGB-1 promote expansion of Th17-like cell through activation of Stat3

After confirming origination of Th17-like cells, we asked how Th17-like cells expanded in inflammatory microenvironment. Previous studies reported that various components, including cytokines (such as IL-6, IL-1β, IL-23 and TNF-α [16,27], etc) and damage-associated molecule patterns (DAMP) (such as HMGB-1 [28,29]), could shape the phenotype and function of Tregs in various diseases. Through cytometric bead array (CBA) and ELISA, we explored significant upregulation of IL-6, IL-1β and HMGB-1 in peripheral blood of active SLE patients (Supplemental Fig. 2C). Consistently, we also confirmed extremely high levels of HMGB-1, IL-6 and IL-1β in kidney homogenates of lupus mice (data not shown). To check their effects on shaping Treg, freshly isolated peripheral $CD4^+CD25^{high}CD127^{low}$ cells were stimulated with IL-6, IL-1β or HMGB-1 in the presence of IL-2, as well as αCD3. To our surprise, not only IL-6, but also HMGB-1 possessed the competence to induce cultured cells to express IL-17 in a dose dependent manner. Moreover, HMGB-1, together with IL-6, rather than IL-1β, dramatically induced IL-17 expression (Supplemental Fig. 3A and Fig. 4A). These data suggested a critical role of HMGB-1 and IL-6 on induction of Th17-like cells.

We next want to know the signal mechanism of increasing of Th17-like cell in active SLE patients. Consistent with the claim that stat3 is necessary to produce IL-17 and RORγt [30], significant higher level of phosphorylated stat3 was found in $Helios^-Foxp3^+CD4^+$ cells of freshly isolated PBMC in patients with active SLE, compared with the same cells from HD (Supplemental Fig. 3B and Fig. 4B). To investigate the impacts of HMGB-1 and IL-6 on phosphorylation of stat3, isolated $CD4^+CD25^{high}CD127^{low}$ cells were rested in FCS-free medium for 2 h, followed by stimulation with HMGB-1 and IL-6 in the presence of αCD3. Data showed that cooperation of HMGB-1 and IL-6 significantly induced phosphorylation of stat3 (Supplemental Fig. 3C and Fig. 4C). To set up direct linkage between stat3 and Th17-like cells, we observed the impact of s31–201, a stat3 specific inhibitor [31], on differentiation of Th17-like cells. S31–201-pretreated $CD4^+CD25^+CD127^{low}$ cells failed to produce IL-17 upon stimulation (Fig. 4D). Collectively, data above reflects a critical role of stat3 activation in differentiation of Th17-like cells.

3.5. Th17-like cells from patients with active SLE respond to stimulation of autoantigen

$Foxp3^+CD4^+CD127^{low}$ Treg cells have a high affinity to autoantigens, but are hypo-responsive upon stimulation of autoantigens [32,33]. We hypothesized that Th17-like cells also have affinity to autoantigens but lose regulatory function during the progress of disease. To investigate the response of Th17-like cells upon stimulation of autoantigen, isolated Th17-like cells, which were defined as $CD4^+CD25^+CCR6^+$ cells with high expression level of IL-17 (Fig. 5A and B), were co-cultured with autoantigens-treated monocyte-derived dendritic cells, as well as recombinant IL-2 for 3 days. Notably, $CD4^+CD25^+CCR6^+$ cells from patients with active SLE proliferated to a great extent than those cells from HD did (Fig. 5C). As for $CD4^+CD25^+CCR6^-$ cells, there was no significant difference of proliferate level between patients and HD (Fig. 5C). We also set up Treg suppression assay with $CD4^+CD25^+CCR6^+$ cells from peripheral $CD4^+$ T cells of active SLE patients and HD. Cells from healthy peripheral

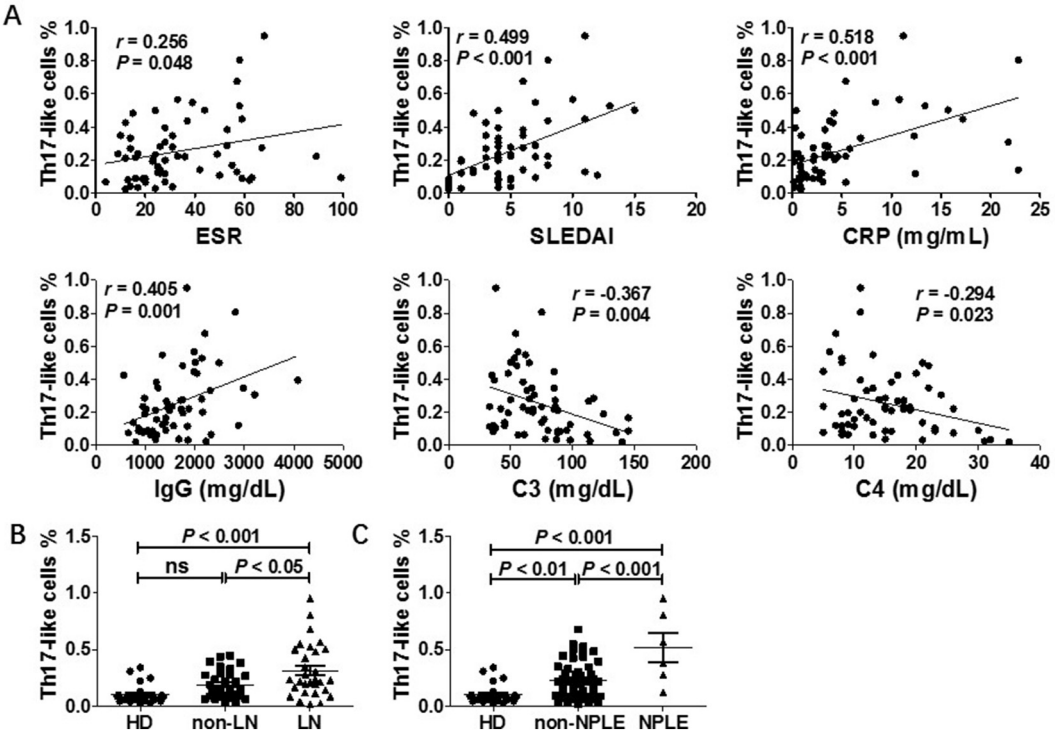

Fig. 6. Positive correlation between Th17-like cells and SLE disease activity. (A) Correlation between the percentage of IL-17-producing Treg in $CD4^+$ T cells and clinical features of patients with SLE was analyzed by Spearman's rank correlation test. Th17-like cells were positively correlated with ESR, CRP, SLEDAI and total IgG in patients with SLE. Th17-like cells were negatively correlated with serum C3 and C4 in patients with SLE. (B) The percentage of Th17-like cells in total $CD4^+$ T cells was compared among HD (n = 27), patients with LN (n = 30), and patients without LN (n = 30). (C) The percentage of Th17-like cells in total $CD4^+$ T cells was compared among HD (n = 27), patients with NPLE (n = 6), and patients without NPLE (n = 54).

blood showed suppressive activity on $CD4^+CD25^-$ responder T-cell (Teff) proliferation. Contrary to cells from healthy population, Th17-like cells from active SLE patients showed decreased suppressive property of Teff cells proliferation (Fig. 5D). Since we found that HMGB-1 and IL-6 promote Treg to express IL-17, we then tested the suppressive capacity of Treg stimulated in vitro by HMGB-1 and IL-6. In line with our anticipation, HMGB-1 and IL-6 impair the regulatory function of Treg cells (Supplemental Fig. 3D). Taken together, these data support our idea that Th17-like cell represents a middle state in which Treg cell converts into Th17 cell.

3.6. Association between Th17-like cells and SLE clinical features

To determine clinical relevance of Th17-like cells in SLE, proportion of these cells among total $CD4^+$ T cells in peripheral blood was measured in a cohort of 53 patients with SLE. The percentage of Th17-like cells was positively associated with ESR, CRP, and total IgG (Fig. 6A). There were also weak but significantly negative correlations between this subset and circulating complement C3 and C4 levels (Fig. 6A). No significant correlation was found with patient age, disease duration, RF, total IgM, total IgA (data not shown). We also checked the relationship between Th17-like cells and major organ lesions, such as lupus nephritis (LN) and neuropsychiatric lupus (NPLE). As shown in Fig. 6B and C., patients with LN or NPLE had striking higher levels of circulating Th17-like cells than patients without relevant organ lesion or healthy controls did. These results support our hypothesis that Th17-like cell plays a pathogenic role in SLE.

4. Discussion

This study provided new evidence for Treg plasticity, and suggested the predisposition of transformation of Treg into Th17 during the progress of SLE. By analysis of clinical features and cytokines expression in circulating $Foxp3^+CD4^+$ Treg cells, we found that induction of Th17-like cells was associated with the disease activity of SLE. Although Th17-like cells were identified in both healthy people and patients, it remains debatable whether the cells originate from $Foxp3^+$ Treg or $Foxp3^-$ effector cell. Main reason for the debate is that Th17-like cells possess phenotypical and functional characters of both Treg cell and effector T cells. Here, our data from both human and mice cells strongly suggests that this subpopulation is induced from canonical $Foxp3^+$ Treg cells during the progress of SLE.

Previous researches divided $Foxp3^+$ Treg cell into two subsets according to the expression of Helios, which is a member of the Ikaros gene family of transcription factors. Helios expression was once associated with thymus-derived Treg in mice, but more recently studies showed that Helios can be induced to express in periphery [22,26]. We checked expression level of Helios in Th17-like cells, and confirmed that almost all Th17-like cells were Helios negative. This is consistent with previous finding that losing Helios expression was associated with instability of Treg [14]. Additionally, epigenetic analysis showed that the methylation level of Foxp3 locus in Th17-like cells is comparable to that of $Helios^-$ Tregs, but much higher than that of $Helios^+$ Treg. Thus, we proposed that Th17-like cells might be transformed from $Helios^-$ Treg.

Converting Treg cells towards Th17-like cells reflected complicated inflammatory condition in SLE patients. Our data showed that cooperation of inflammatory cytokines IL-6 and DAMP molecules HMGB-1 strongly drive the conversion of Treg. A critical role of IL-6 in inducing IL-17 and canonical Th17 has been wildly proven [34]. HMGB-1, a kind of nonhistone and highly mobile DNA-binding protein, which is from apoptosis cells, was reported to regulate immunoreaction through Toll like receptor (TLR)2 and TLR4 [35,36]. In active patients, because of clearance deficiency of apoptosis debris, accumulation of various DAMPs will finally lead to uncontrollable inflammation. Such DAMPs, including HMGB-1, not only incite serious immunoreaction, but also

destroy regulatory function. Our data clearly suggests that HMGB-1 alter phenotype of Treg cells, and impair their regulatory function.

Consistent with the finding of IL-17 induction, RORγt, the critical transcript factor of Th17 cells [37], was also found increase in freshly isolated Th17-like cells from SLE active patients and in vitro HMGB-1 and IL-6- treated Treg cells. Previous studies have demonstrated that Treg cells appropriate partial or "aborted" forms of the transcriptional programs of target Th cells by expressing their master transcription factors, such as T-bet for Th1 cells and IRF4 for Th2 cells, and co-opting their function [38,39]. RORγt expressed in Treg cells results in production of IL-17, as well as inhibition of Th17- and Th2-mediated autoimmune reaction [40,41]. Whereas under physiological conditions, such partial Th cell programming remains restrained. Such restraint disappears under the influence of heightened proinflammatory signaling and thus leads to pathogenic reprogramming of Treg cells into Th17 cells. These converted cells may directly contribute to disease, as evidenced by the middle status. Th17-like cells, which lose immunoregulatory function, but show robust response to stimulation of autoantigens.

It seemed that the conversion of Treg to Th17 cells driven by inflammatory stimulus can be stopped or even reversed, because we found that stat3 is critical to such conversion. Previous studies have shown that activation of stat3, combined with TGF-β, leads to increased expression of orphan nuclear receptors RORγ and RORα, signature transcription factors for Th17 cell [42]. Here, we also showed that both HMGB-1 and IL-6 can mediate phosphorylation of stat3, blocking which inhibited IL-17 expression of Treg. This data suggests a critical role of stats signaling in conversion between Treg and Th17. Regulating the level and activation of stats3 is beneficial for retaining homeostasis of Treg.

The role of Treg cells in SLE is still largely unknown. However, as human Treg cells can be used in clinical trials as an immunotherapy, it is essential to understand their behaviors in inflammatory environments. In this study, we demonstrate that IL-17-producing Treg cells are influenced by environmental factors to exhibit Teff function and phenotype. This cell subset may be a good target for future diagnosis and therapy of SLE, because its level and phenotype are closely related to clinical appearance, such as SLEDAI and organ lesion. However, it is important to keep in mind that majority of Treg cells studies in SLE, including this one, are limited by peripheral blood compartment and may not directly reflect the states of Treg cells in tissues with end-organ damage in SLE. Also, because of species difference, results of animal experiments cannot fully reflect real state of human diseases. Further studies will focus on this subset in situ in tissue samples from SLE patients, which may benefit uncovering mechanisms underlying this conversion, understanding function of plastic Foxp3$^+$ T cells, and leading to development of new therapeutic strategies for SLE.

Supplementary data to this article can be found online at https://doi.org/10.1016/j.clim.2019.108267.

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Appendix A. Supplementary data

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