CCR6$^+$ Th cell distribution differentiates systemic lupus erythematosus patients based on anti-dsDNA antibody status

Wei Zhong[1,*], Zhenyu Jiang[1,*], Jiang Wu[2], Yanfang Jiang[3,4] and Ling Zhao[1]

[1] Department of Rheumatology, First Hospital, Jilin University, Changchun, China
[2] College of Electrical Engineering and Instrumentation, Jilin University, Changchun, China
[3] Genetic Diagnosis Center, First Hospital, Jilin University, Changchun, China
[4] Key Laboratory of Zoonosis Research, Ministry of Education, First Hospital, Jilin University, Changchun, China
[*] These authors contributed equally to this work.

ABSTRACT

Background. Systemic lupus erythematosus (SLE) disease has been shown to be associated with the generation of multiple auto-antibodies. Among these, anti-dsDNA antibodies (anti-DNAs) are specific and play a pathogenic role in SLE. Indeed, anti-DNA$^+$ SLE patients display a worse disease course. The generation of these pathogenic anti-DNAs has been attributed to the interaction between aberrant T helper (Th) cells and autoimmune B cells. Thus, in this study we have investigated whether CCR6$^+$ Th cells have the ability to differentiate SLE patients based on anti-DNA status, and if their distribution has any correlation with disease activity.

Methods. We recruited 25 anti-DNA$^+$ and 25 anti-DNA$^-$ treatment-naive onset SLE patients, matched for various clinical characteristics in our nested matched case-control study. CCR6$^+$ Th cells and their additional subsets were analyzed in each patient by flow cytometry.

Results. Anti-DNA$^+$ SLE patients specifically had a higher percentage of Th cells expressing CCR6 and CXCR3. Further analysis of CCR6$^+$ Th cell subsets showed that anti-DNA$^+$ SLE patients had elevated proportions of Th9, Th17, Th17.1 and CCR4/CXCR3 double-negative (DN) cells. However, the proportions of CCR6$^-$ Th subsets, including Th1 and Th2 cells, did not show any association with anti-DNA status. Finally, we identified a correlation between CCR6$^+$ Th subsets and clinical indicators, specifically in anti-DNA$^+$ SLE patients.

Conclusions. Our data indicated that CCR6$^+$ Th cells and their subsets were elevated and correlated with disease activity in anti-DNA$^+$ SLE patients. We speculated that CCR6$^+$ Th cells may contribute to distinct disease severity in anti-DNA$^+$ SLE patients.

INTRODUCTION

Systemic lupus erythematosus (SLE) is a multi-organ autoimMune disorder that can affect the skin, joints, kidneys and central nervous system (*Kaul et al., 2016*). These effects are usually mediated by generation of auto-antibodies and immune complexes. Indeed,

SLE has been observed to be associated with more than 100 different auto-antibodies including anti-nuclear antibody (ANA), anti-dsDNA antibody (anti-DNA), anti-Sm antibody, anti RNP antibody, anti Ro antibody, anti La, and anti-phospholipid antibody (*Sherer et al., 2004*). Among these, anti-DNA is a crucial pathogenic SLE factor and its production leads to SLE development (*Kaul et al., 2016*). In addition, it is one of the 11 criterion used for SLE diagnosis and has been observed to play a prominent role in SLE, as highlighted by the observation that two-thirds of SLE patients have detectable anti-DNA (*Fattal et al., 2014*) levels. The identification of anti-DNA in SLE patients very early—and even before the onset of disease—further highlights its role in a clinically overt disease (*Arbuckle et al., 2003*; *Kaul et al., 2016*). Typically, anti-DNA$^+$ SLE patients display a severe disease course, and suffer from significant type III/IV lupus nephritis (LN) (*Conti et al., 2015*; *Mavragani et al., 2015*). These patients also have worse treatment outcomes in comparison to anti-DNA$^-$ patients (*Lazarus et al., 2012*; *Linnik et al., 2005*). One study has demonstrated a direct link between the development of anti-DNA and the HLA-DRB1 * 1501(DR2) allele polymorphism in SLE patients (*Podrebarac, Boisert & Goldstein, 1998*), implying a breakdown of immunological tolerance against self-antigens, most likely through aberrant MHC class II dependent activation of auto-reactive CD4$^+$ T cells. During normal immune response to foreign antigens, there is inherently a production of pathogenic auto-antibodies in the germinal centers of follicles (*Ray, Putterman & Diamond, 1996*). A particular population of autoimmune CD4$^+$ T cells usually drive the production of pathogenic anti-DNA by cognate interactions with the autoimmune B cells found in SLE (*Datta, 1998*). In SLE patients, aberrant T cell activation, coupled with nonspecific activation of B cells, leads to overproduction of a plethora of auto-antibodies. Specifically, pathogenic anti-DNA are of an IgG type and are used as a quintessential clinical biomarker. These findings also suggest that anti-DNA-producing B cells have undergone isotype switching, dependent upon the presence of T cells (*Pisetsky, 2016*).

Generally T cells and in particular CD4$^+$ cells initiate and perpetuate autoimmunity. Infiltrating activated T cells have been observed in tissue samples collected from organs affected by lupus (*Crispin et al., 2008*; *Enghard et al., 2009*; *Robak et al., 2001*). In addition, cytokines expressed by T cells, including TNF-α and IL-17, both of which play an active role in autoimmunity, are increased in SLE patients (*Vincent et al., 2013*). Moreover, the chemokine receptor, CCR6, expressed on Th cells, has been implicated in mediating the recruitment of IL-17 producing cells in glomerulonephritis (*Koga et al., 2016*; *Turner et al., 2010*).

Such chemokine receptors have typically been used to characterize memory Th cell subsets, with different effector and migratory functions (*Sallusto, Mackay & Lanzavecchia, 2000*). Due to heterogeneity, CCR6$^+$ Th cell can be distinguished into several subpopulations, such as IL-17A and IL-22 producing CCR6$^+$ T cell subpopulations. CCR6$^+$ cells with Th17 characteristics have a CCR4$^+$CCR10$^-$CXCR3$^-$ phenotype (*Duhen et al., 2009*; *Trifari et al., 2009*; *Van Hamburg et al., 2013*), while those with Th22 characteristics display a CCR4$^+$CCR10$^+$ phenotype (*Duhen et al., 2009*; *Mahnke, Beddall & Roederer, 2013*). Interestingly, Th17.1 cells with CCR6$^+$CCR4$^-$CXCR3$^+$ phenotype can produce both IL-17 and IFN-γ, which were previously thought to be mutually exclusive functional

characteristics (*Acosta-Rodriguez et al., 2007*). In addition, IL-9 producing Th9 cells, which have CCR6$^+$CCR4$^-$ phenotype, has been suggested to play a role in wound healing of pleural mesothelial cells during *M. tuberculosis* infection (*Ye et al., 2012*). However, CCR6$^-$ Th cells, Th1 cells with CCR6$^-$CCR4 $^-$CCR10$^-$CXCR3$^+$ phenotype and producing IFN-γ (*Bonecchi et al., 1998*; *Duhen et al., 2009*), and Th2 cells with CCR6$^-$CCR4$^+$CXCR3$^-$ phenotype, are involved in secreting IL-5, IL-4 and IL-13 chemokines (*Rivino et al., 2004*). Based on the differential expression of CCR6 on Th cells, recent studies have indicated their potential proinflammatory role in the development of autoimmune disorders, including rheumatoid arthritis (*Paulissen et al., 2015a*; *Paulissen et al., 2015b*). It has also been demonstrated that pathogenic Th17 cells expressing CCR6 play a key role in accelerating organ injury in animal models of glomerulonephritis (*Turner et al., 2010*) and arthritis (*Hirota et al., 2007*). In addition, a genetic link has also been reported between CCR6 gene polymorphisms and LN susceptibility (*Zhou et al., 2015*).

Thus, crucial role of anti-DNA in SLE pathogenesis, association of its production with T cell engagement, its involvement along with CCR6$^+$ Th cells in kidney impairment, and differential clinical course between anti-DNA positive and negative patients, prompted us to investigate the differences in Th cell distribution between anti-DNA$^+$ and anti-DNA$^-$ SLE patients. In addition, we also tested the possibility of a correlation between Th cell subsets and disease activity in anti-DNA$^+$ SLE patients.

MATERIALS AND METHODS

Patients and samples

We performed a matched case-control study where 25 anti-DNA$^+$ and 25 anti-DNA$^-$ treatment-naive onset SLE patients were recruited from the in-patient service of the First Hospital of Jilin University, China between January 2016 and January 2017. All patients had clinical symptoms for less than three months. Patients were matched for SLE disease activity score, presence of anti-Sm antibodies, sex, age, and disease duration. SLE diagnosis was conducted according to the revised criteria for the classification of SLE by the American College of Rheumatology (*Hochberg, 1997*; *Tan et al., 1982*), and the disease activity of each patient was assessed based on the SLE disease activity index (SLEDAI) (*Bombardier et al., 1992*). Complicated LN in the patients with renal involvement was defined according to the ACR criteria, where patient typically displayed: (i) persistent urinary protein level of $\geq$0.5 g/day; (ii) presence of active cellular casts; and (iii) biopsy evidence of lupus nephritis (*Hahn et al., 2012*; *Schiffenbauer & Simon, 2004*). However, subjects with a history of systemic sclerosis, myositis or other autoimmune diseases, or had a recent infection or received immunosuppressive or glucocorticoid treatment within the past six months, were excluded. In addition, written informed consent was obtained from individual participants. Authors had access to information that could identify individual participants during and after data collection. The experimental protocol was established based on the Declaration of Helsinki guidelines, and was approved by the Human Ethics Committee of Jilin University, China (approval number: 2016-373).

Data collection

The demographic and clinical characteristics of the subjects, including age and gender at baseline, were obtained from the hospital records. Routine laboratory tests, including determination of complete blood cell counts, concentrations of plasma complement factors (C3, C4), erythrocyte sedimentation rate (ESR), C-reactive protein (CRP) were measured as described previously (*Zhao et al., 2013*). The levels of serum anti-DNA and anti-Sm antibody were measured by indirect immunofluorescence using special kits, according to the instruction of manufacturers (Oumeng, Beijing, China).

Isolation of peripheral blood mononuclear cells (PBMCs)

The peripheral blood (5 mL) was collected from all participants after an overnight fasting, and PBMCs were subsequently isolated by density-gradient centrifugation at 800 g for 30 min using Ficoll-Paque Plus (Amersham Biosciences, Buckinghamshire, UK). The cells were later resuspended at a concentration of 1×10^6 per mL in RPMI-1640 culture medium (Invitrogen, Carlsbad, CA, USA) containing 10% fetal calf plasma.

Flow cytometry analysis

The PBMCs were stained in duplicate with APC-H7-anti-CD4 (Becton Dickinson, San Diego, CA, USA), PerCP-Cy5.5-anti-CD3 (Becton Dickinson), BV510-anti-CCR4 (Becton Dickinson), PE-Cy7-anti-CCR6 (Becton Dickinson), BB515-anti-CCR10 (Becton Dickinson), and PE-CF594-anti-CXCR3 (Becton Dickinson) antibodies in the dark at 4 °C for 30 min. Negative controls were stained with isotype-matched control antibodies (APC-H7-anti-IgG1, PerCP-Cy5.5-anti-IgG1, BV510-anti-IgG1, PE-Cy7-anti-IgG1, BB515-anti-IgG2a, PE-CF594-anti-IgG1). The frequencies of different T cell subsets were examined by flow cytometry analysis on a FACSAria II (Beckton Dickinson, San Diego, CA, USA) and analyzed by FlowJo software for Microsoft (v7.6.2, TreeStar, San Carlos, CA, USA).

Statistical analysis

Statistical analysis of the data was performed using SPSS 21.0 (SPSS, Chicago, IL, USA) software. The quantitative data were represented as individual values, or median (range) of each group. The differences between two groups of patients were analyzed using Wilcoxon matched-pairs signed-ranks test. The correlation analysis was performed using Spearman's rank correlation test. A two-sided P value of <0.05 represented statistical significance.

RESULTS

Demographic and clinical characterization of SLE patients based on anti-DNA status

A total of 25 SLE patients with anti-DNA positive status and another 25 matched patients with negative anti-DNA status were recruited to undertake this study. Their demographic and clinical characteristics are summarized in Table 1. Specifically, anti-DNA$^+$ SLE patients displayed lower concentrations of C3 and C4 proteins ($P = 0.0010$, $P = 0.0150$). However, a higher number of anti-DNA$^+$ SLE patients showed renal impairment ($P = 0.0460$), thereby indicating higher activation of the complement system and subsequent worse

Table 1 Demographic and clinical characteristics of anti-DNA$^+$ and anti-DNA$^-$ treatment-naive patients with onset SLE.

Parameters	anti-DNA$^+$ ($n = 25$)	anti-DNA$^-$ ($n = 25$)
Age (years)	31 (18–61)	27 (17–58)
Gender: female/male	21/4	21/4
SLEDAI	17 (2–36)	19 (7–39)
Positive anti-Sm	14 (56.00%)	10 (40.00%)
ESR (mm/h)	46 (8–90)	55 (3–118)
CRP (mg/L)	9.40 (0.43–51)	6.85 (3.16–192)
C3 (IU/mL)	0.36 (0.12–1.52)*	0.73 (0.17–1.62)
C4 (U/mL)	0.06 (0.00–0.35)*	0.15 (0.02–0.50)
Renal involvement	16 (64%)*	8 (32%)

Notes.
Data shown are median (range) or number of cases.
SLE, systemic lupus erythematosus; anti-DNA, anti-dsDNA antibodies; SLEDAI, SLE disease activity index; ESR, Erythrocyte sedimentation rate; CRP, C-reactive protein. Normal values: ESR: 0–15 mm/h, CRP: 0–3 mg/L, C3: 0.9–1.8 units/mL,C4: 0.1–0.4 units/mL.
$^*P < 0.05$ versus anti-DNA$^-$ SLE.

prognosis. Interestingly, many of the other parameters studied in these patients showed no significant differences based on anti-DNA status.

Anti-DNA$^+$ SLE patients revealed increased percentages of CCR6$^+$ and CXCR3$^+$ Th cells

Next, we analyzed the Th cell population in SLE patients from both groups, based on chemokine expression profile, as different chemokine receptors are expressed on the surface of T cells. Specifically, the expression of CCR4, CCR6, CCR10 and CXCR3 chemokines was analyzed on T cells (CD3$^+$CD4$^+$) isolated from peripheral blood mononuclear cells (PBMCs) of these patients. Flow cytometry analysis illustrated significantly higher proportions of CCR6$^+$ (C) ($P = 0.0061$) and CXCR3$^+$ (E) ($P = 0.0114$) T cells in anti-DNA$^+$ SLE patients in comparison to anti-DNA$^-$ SLE patients. However, no significant differences were observed in Th cells expressing CCR4 (B) ($P = 0.1785$) and CCR10 (D) ($P = 0.6865$) between these two groups of patients, as shown in Fig. 1. In addition, percentage of total CCR6$^+$ Th cells correlated positively with anti-DNA titer in SLE patients ($r = 0.4668$ $P = 0.0006$; Table 2), but no correlations were observed between anti-DNA titers and CCR4$^+$, CCR10$^+$, CXCR3$^+$ T cells ($P > 0.05$; Table 2).

Anti-DNA$^+$ SLE patients specifically showed elevated proportions of CCR6$^+$ Th9, Th17, Th17.1 and CCR6$^+$CCR4$^-$CXCR3$^-$ Th cells subsets

In addition, we also analyzed the different subsets of Th cells based on the enrichment of different chemokine expression in SLE patients from both groups. CD3$^+$CD4$^+$ T cells were gated based on chemokine expression to identify Th1, Th2, Th9, Th17, Th22 or a Th17.1 phenotypes, as shown in Fig. 2A. CCR6$^+$CCR4$^+$CCR10$^+$ phenotype depicted Th22 cells, while CCR6$^+$CCR4$^+$CXCR3$^-$CCR10$^-$ phenotype represented Th17 cells, and CCR6$^+$ CCR4$^-$ phenotype established Th9 cell subset. Also, Th17.1 cells with Th1 and Th17 profile were gated as CCR6$^+$CCR4$^-$CXCR3$^+$CCR10$^-$. In addition, we also measured the
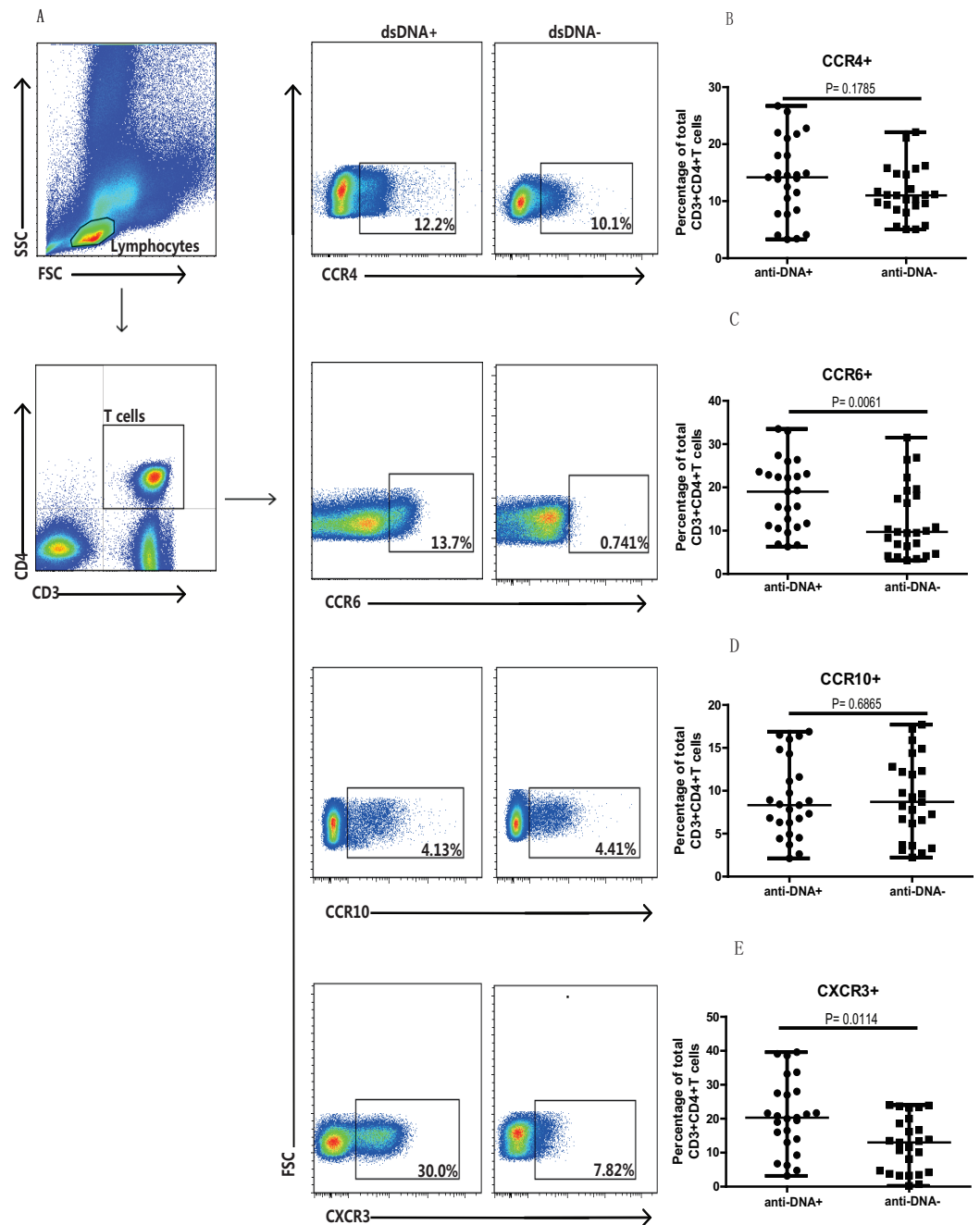
Figure 1 **Analysis of circulating CD3$^+$ & CD4$^+$ T cells based on chemokine expression in SLE patients.** Peripheral blood mononuclear cells (PBMCs) collected from new onset anti-DNA$^+$ and anti-DNA$^-$ SLE patients at baseline ($n = 25$ for each group) were analyzed by flow cytometry. (A) represented the gating strategy, while (B–E) showed the percentages of CCR4$^+$, CCR6$^+$, CCR10$^+$ and CXCR3$^+$ Th cells, respectively in anti-DNA$^+$ and anti-DNA$^-$ SLE patients.

Full-size 🖾 DOI: 10.7717/peerj.4294/fig-1

Table 2 Correlation of Th cell populations (%CD3$^+$CD4$^+$ T cells) with anti-DNA titers in SLE patients.

Populations	r	P value
CCR4$^+$ T cells	0.1734	0.2286
CCR6$^+$ T cells*	0.4668	0.0006
CCR10$^+$ T cells	0.0221	0.8789
CXCR3$^+$ T cells	0.2729	0.0552
Th1 cells	0.2449	0.0865
Th2 cells	0.0478	0.7406
Th9 cells*	0.4218	0.0023
Th17 cells*	0.3608	0.0101
Th17.1 cells*	0.4192	0.0024
Th22 cells	0.0682	0.6377
CCR6$^+$CCR4$^+$CXCR3$^+$ T cells	0.1972	0.1700
CCR6$^+$CCR4$^-$CXCR3$^-$ T cells*	0.4510	0.0010

Notes.
SLE, systemic lupus erythematosus; anti-DNA, anti-dsDNA antibodies.
All analyses were performed using Spearman's rank correlation test.
$^*P < 0.05$.

percentages of unclassified CCR6$^+$ T cells, including CCR4/CXCR3 double-positive (DP) (CCR6$^+$CCR4$^+$CXCR3$^+$ phenotype) and double-negative (DN) (CCR6$^+$CCR4$^-$CXCR3$^-$ phenotype) Th cells, as shown in Fig. 3A. Our overall analysis of these different Th cell subsets in SLE patients based on anti-DNA status indicated that CCR6$^+$ Th subsets, such as Th9 ($P = 0.0006$) (Fig. 2B), Th17 ($P = 0.0274$) (Fig. 2D), Th17.1 ($P = 0.0084$) (Fig. 2E) and CCR6$^+$CCR4$^-$CXCR3$^-$ Th cells ($P = 0.0179$) (Fig. 3C), were significantly increased in anti-DNA$^+$SLE patients. However, no significant difference was observed in the distribution of CCR6$^+$ Th22 ($P = 0.4675$) (Fig. 2C) and CCR6$^+$CCR4$^+$CXCR3$^+$ Th cells ($P = 0.2158$) (Fig. 3B) between anti-DNA$^+$ and anti-DNA$^-$ SLE patients. Positive correlations of anti-DNA titer were observed with Th9 ($r = 0.4218$, $P = 0.0023$), Th17 ($r = 0.3608$ $P = 0.0101$), Th17.1 ($r = 0.4192$ $P = 0.0024$) and CCR6$^+$CCR4$^-$CXCR3$^-$ Th ($r = 0.4510$ $P = 0.0010$; Table 2) cells in SLE patients. Similarly, CCR6$^-$ Th subpopulations, such as Th1 ($P = 0.1389$) (Fig. 2G) and Th2 ($P = 0.8930$) (Fig. 2F), which were gated as CCR6$^-$CCR4$^-$CXCR3$^+$CCR10$^-$ and CCR6$^-$CCR4$^+$CXCR3$^-$CCR10$^-$, respectively (Fig. 2A), did not show any significant differences between patients of both groups, and also showed no correlation with anti-DNA titer in these patients ($P > 0.05$; Table 2).

## Elevated percentage of CCR6$^+$ Th9 subsets correlated with anti-DNA status in SLE patients

We next examined if higher levels of CCR6$^+$ Th9, Th17, Th17.1 and CCR4/CXCR3 DN Th cell populations in anti-DNA$^+$ SLE patients were due to the overall increase of CCR6$^+$ Th cells (Fig. 1C) or whether they specifically correlate with anti-DNA status. Thus, we plotted all CCR6$^+$ Th cell subpopulations as a proportion of total CCR6$^+$ Th cells in each group. Interestingly, the proportions of all CCR6$^+$ subpopulations were comparable between anti-DNA$^+$ and anti-DNA$^-$ patients, except Th9 cells (A) ($P = 0.0207$), which
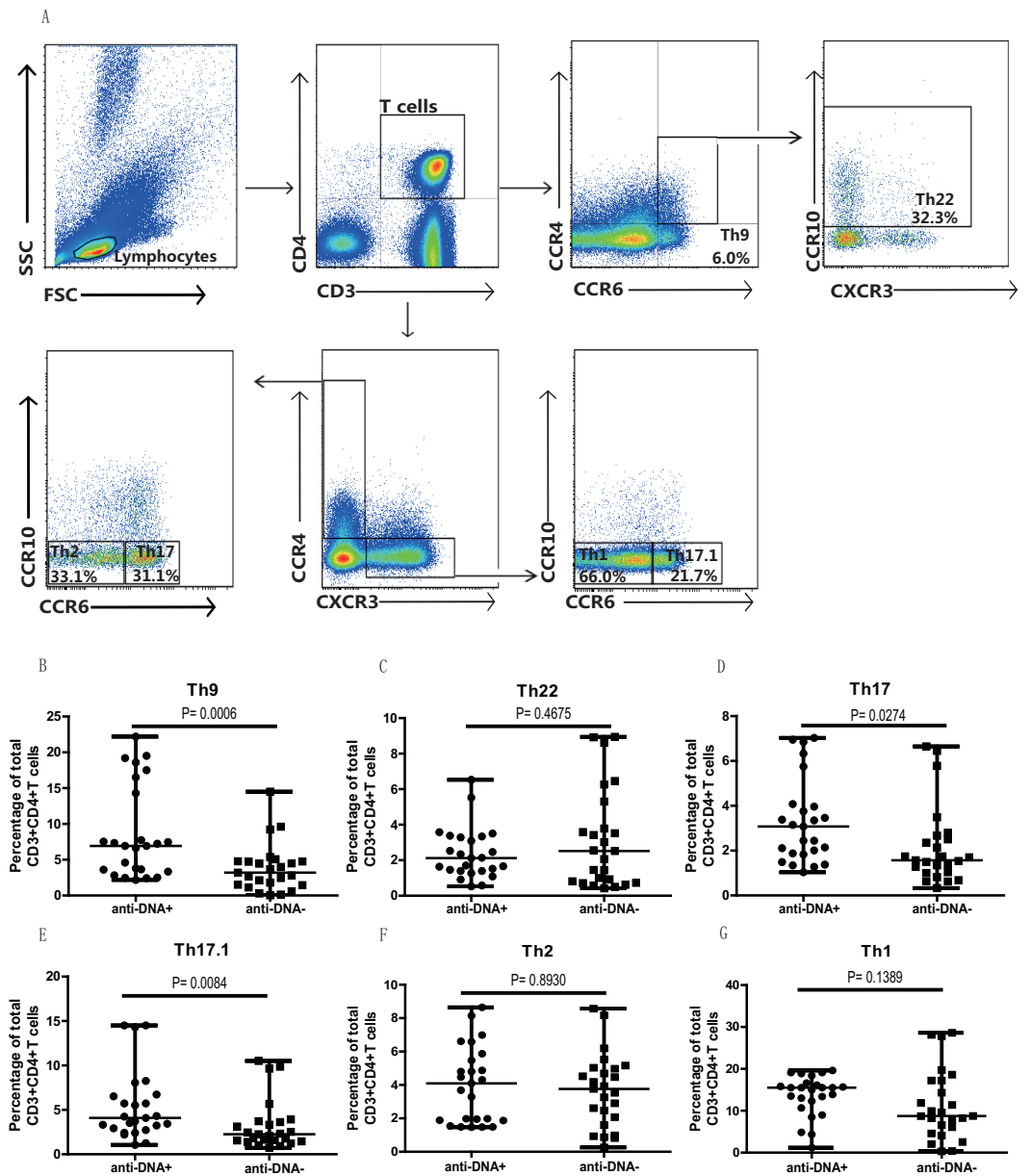
Figure 2 Analysis of the percentages of various circulating Th cell subsets in SLE patients. PBMCs collected from new onset anti-DNA$^+$ and anti-DNA$^-$ SLE patients ($n = 25$ in each group) at baseline were analyzed by flow cytometry for the percentages of different Th subset. (A) depicted the gating strategy for these different Th subsets, while (B)–(G) showed the comparative quantitative analysis of Th9 (CCR6$^+$CCR4$^-$), Th22 (CCR6$^+$CCR4$^+$CCR10$^+$), Th17 (CCR6$^+$CCR4$^+$CXCR3$^-$CCR10$^-$), Th17.1 (CCR6$^+$CCR4$^-$CXCR3$^+$CCR10$^-$), Th2 (CCR6$^-$CCR4$^+$CXCR3$^-$CCR10$^-$), and Th1 (CCR6$^-$CCR4$^-$CXCR3$^+$CCR10$^-$) subsets in anti-DNA$^+$ and anti-DNA$^-$ SLE patients.

Full-size 🖼 DOI: 10.7717/peerj.4294/fig-2
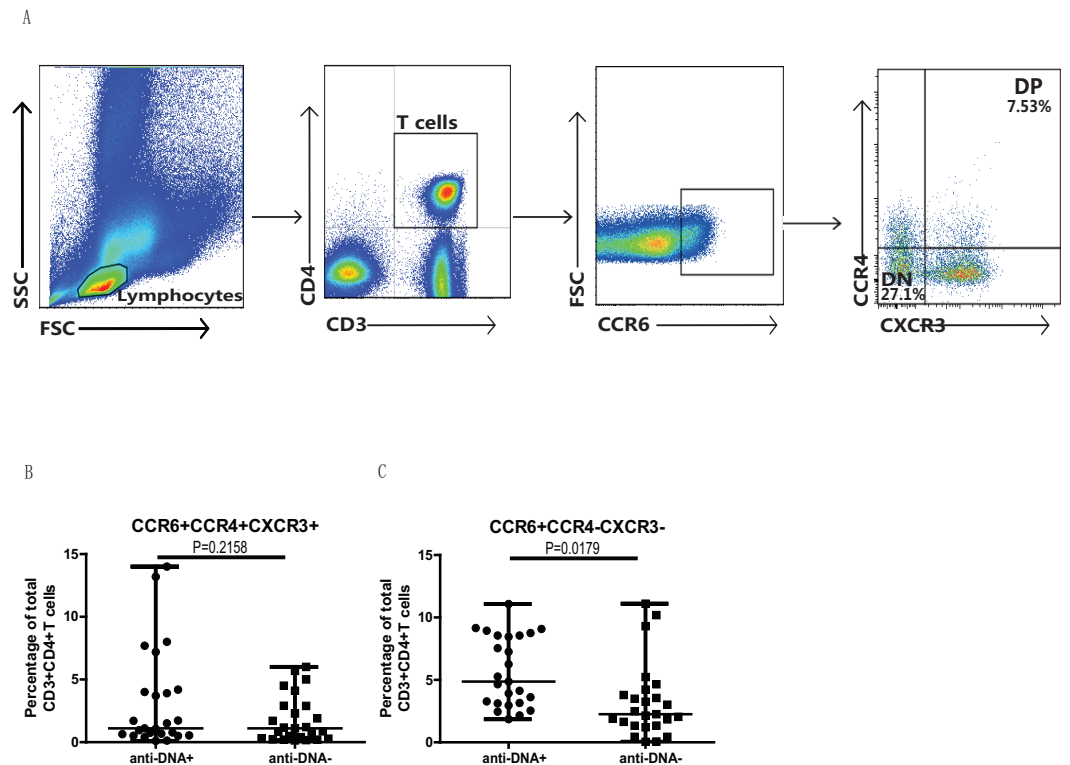
Figure 3 **Analysis of circulating CCR4/CXCR3 DP and CCR4/CXCR3⁻ DN CCR6⁺ T cells in SLE patients.** PBMCs collected from new onset anti-DNA⁺ and anti-DNA⁻ SLE patients ($n = 25$ for each group) at baseline were analyzed by flow cytometry for the percentages of unclassified CCR6⁺ Th subsets, CCR6⁺CCR4⁺CXCR3⁺, double positive(DP) and CCR6⁺CCR4⁻CXCR3⁻, double negative (DN) Th cells. (A) represented the gating strategy, while (B) and (C) showed the comparative quantitation of these Th cell subsets in anti-DNA⁺ and anti-DNA⁻ SLE patients.

Full-size 🖾 DOI: 10.7717/peerj.4294/fig-3

were significantly higher in anti-DNA⁺ patients than anti-DNA⁻ patients, as shown in Fig. 4. This observation indicated that elevated Th9 cell subset might have some functional correlation with anti-DNA status.

Clinical indicators correlated with CCR6⁺ Th cell population in anti-DNA⁺ SLE patients

Finally, we tested the correlation between different SLE clinical indicators (disease activity) and CCR6⁺ Th cell subsets. We observed that the percentage of total CCR6⁺ Th cells correlated positively with ESR ($r = 0.4254$ $P = 0.0340$; Fig. 5A) in anti-DNA⁺ SLE patients. Further analysis of the correlation of specific subsets of CCR6⁺ Th cell with clinical parameters suggested that Th9 ($r = 0.5387$ $P = 0.0055$; Fig. 5B), Th22 ($r = 0.4680$ $P = 0.0183$; Fig. 5C), Th17 ($r = 0.4967$ $P = 0.0115$; Fig. 5D), CCR6⁺CCR4/CXCR3 DP ($r = 0.4438$ $P = 0.0262$; Fig. S1A), and CCR6⁺CCR4/CXCR3 DN ($r = 0.6383$ $P = 0.0006$; Fig. S1C) subsets positively correlated with ESR. In addition, Th17 cells ($r = 0.6044$ $P = 0.0014$; Fig. 5E), CCR6⁺CCR4/CXCR3 DP ($r = 0.7740$ $P < 0.0001$; Fig. S1B), and CCR6⁺CCR4/CXCR3 DN ($r = 0.6453$ $P = 0.0005$; Fig. S1D)
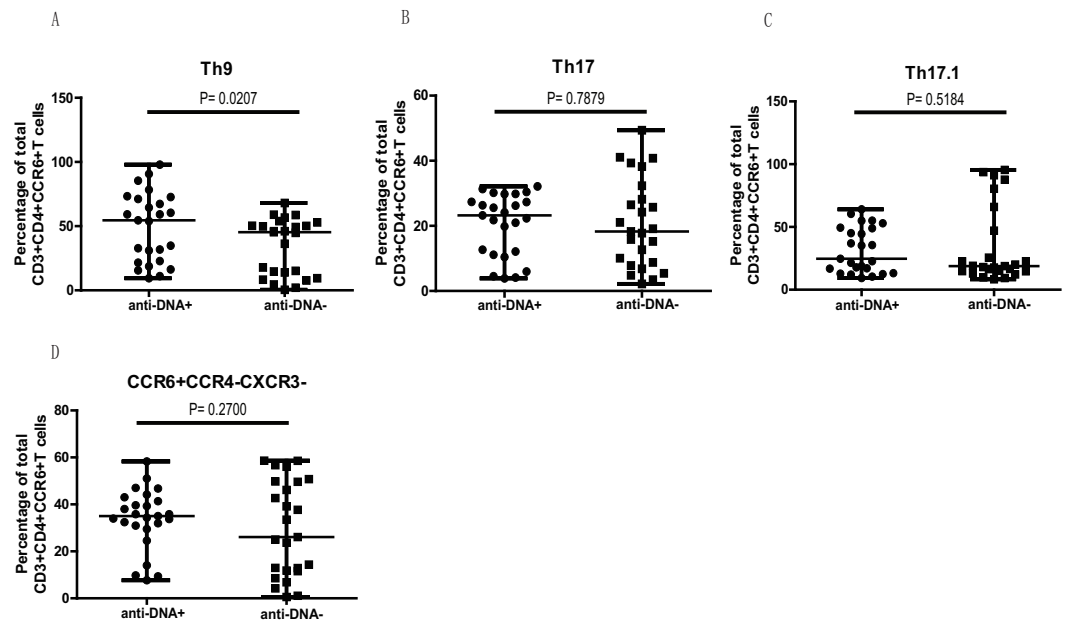
Figure 4 Comparative analysis of the circulating Th9, Th17, Th17.1 and CCR6$^+$CCR4$^-$CXCR3$^-$ Th subsets in SLE patients. The quantitative analysis of the percentages of circulating Th9, Th17, Th17.1 and CCR6$^+$CCR4$^-$CXCR3$^-$ Th cells in anti-DNA$^+$ and anti-DNA$^-$ SLE patients.

Full-size ⊡ DOI: 10.7717/peerj.4294/fig-4

subsets also showed positive correlation with SLEDAI. Interestingly, Th17.1 cell subset numbers showed an inverse correlation with C3 levels ($r = -0.4238$ $P = 0.0347$; Fig. 5F). Importantly, the anti-DNA$^-$ SLE patients revealed no significant correlation between the frequency of CCR6$^+$ Th cell subsets and any disease indicator ($P > 0.05$; Fig. 5 and Fig. S1).

DISCUSSION

In this study, we have specifically focused to assess the distribution of CCR6$^+$ Th cells in anti-DNA$^+$ SLE patients. Our results clearly demonstrated that anti-DNA$^+$ onset SLE patients have higher levels of peripheral CCR6$^+$ Th cells in comparison to anti-DNA$^-$ patients. Further analysis of additional Th cell subsets confirmed elevated percentages of Th9, Th7, Th17.1 and CCR4/CXCR3 DN CCR6$^+$ Th cells. Interestingly, these cell subsets showed positive correlations of anti-DNA titers in SLE patients. However, we identified that the elevated percentages in most of the Th cell subsets were due to an elevated proportion of total CCR6$^+$ Th cells, except for Th9 subset. This specific observation highlighted the importance of Th9 cell subset in anti-DNA$^+$ SLE patients. In addition, the percentages of CCR4/CXCR3 DP CCR6$^+$ Th and Th22 cells were similar in anti-DNA$^+$ and anti-DNA$^-$ SLE patients. However, our findings revealed an increase in the percentage of Th1 and Th2 cells in anti-DNA$^+$ SLE patients in comparison to anti-DNA$^-$ patients. However, this difference was not statistically significant. Importantly, anti-DNA$^+$ SLE patients specifically
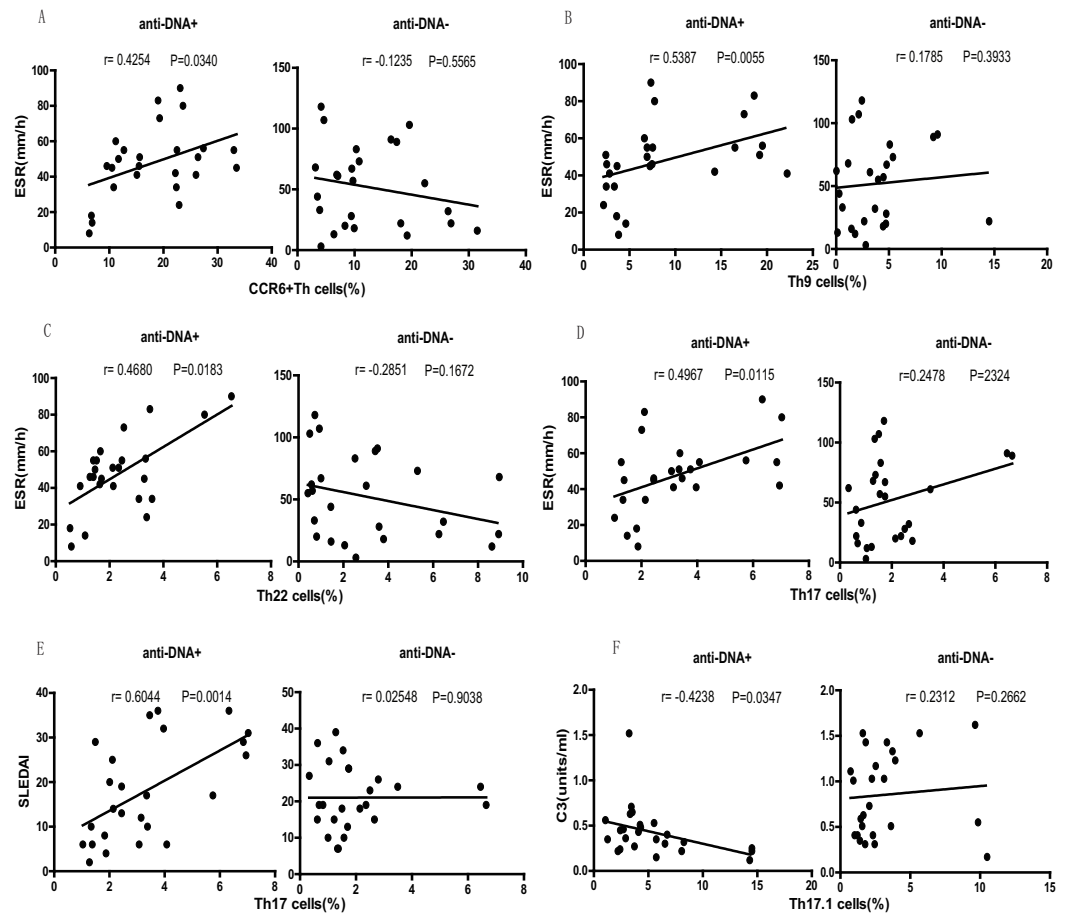
Figure 5 Correlational analysis between circulating CCR6$^+$ Th subsets and clinical parameters of anti-DNA$^+$ and anti-DNA$^-$ patients. (A–D) depicted the correlation analysis of ESR value with circulating CCR6$^+$ Th cells, Th9 cells, Th22 cells and Th17 cells respectively in anti-DNA$^+$ and anti-DNA$^-$ SLE patients. (E) showed the similar correlation between SLEDAI value and the percentage of circulating Th17 cells, while (F) represented the correlation between C3 value and the percentage of circulating Th17.1 cells. All analyses were performed using Spearman's rank correlation test.

Full-size 🖾 DOI: 10.7717/peerj.4294/fig-5

showed a correlation between the percentages of CCR6$^+$ Th cells and their subsets and clinical indicators. However, anti-DNA$^-$ patients showed no correlation between clinical indicators and CCR6$^+$ or CCR6$^-$ Th cells.

Thus, our data suggested that the distribution of CCR6$^+$ Th cell populations correlates with anti-DNA status, and is consistent with previously published studies, which linked anti-DNA$^+$ status of SLE patients with Th cells (*Datta, 1998*; *Pisetsky, 2016*; *Podrebarac, Boisert & Goldstein, 1998*; *Ray, Putterman & Diamond, 1996*). Anti-DNA positivity has also been shown to be strongly associated with MHC class II-restricted HLA-DRB1 ∗1501(DR2) allele, and anti-DNAs are synthesized due to cognate interactions between aberrantly activated T cell and autoimmune B cells. Thus, one can speculate that specific T cells might play a functional role in anti-DNA$^+$SLE patients (*Mohan et al., 1993*). In this context, we found a positive correlation between the elevated percentage of CCR6$^+$

Th populations and ESR in anti-DNA$^+$ SLE patients, thereby supporting the notion that these cells indeed may play a critical role in disease activity specifically, in anti-DNA$^+$ SLE patients. It is important to highlight that the correlation of CCR6$^+$ Th cells with disease activity was not observed in anti-DNA$^-$ SLE patients.

A significant association between CCR6 gene polymorphism and susceptibility to LN in SLE patients has also been reported (*Zhou et al., 2015*). Furthermore, it has been identified that CCR6 chemokine expressed by the effector/memory CD4$^+$ T cells plays a role tissue damage in SLE patients, including LN or neuropsychiatric SLE patients, and probably functions through CCR6/CCL20 axis at the inflamed sites (*Koga et al., 2016*). These observations tentatively indicate that CCR6$^+$ Th cells might be engaged in the aggravation of inflammatory reactions, and thus contributes to worse disease outcomes in anti-DNA$^+$ SLE patients. Higher levels of CCR6$^+$ T cells might play a role in risk-stratification of SLE patients, especially those with positive anti-DNA. It is well established that CCR6$^+$ Th subpopulations display considerable heterogeneity and various subpopulations can be identified based on the differential expression of chemokine receptors. However, the contribution of these CCR6$^+$ Th subsets in the disease severity of anti-DNA$^+$ SLE patients has not been fully investigated.

Among various CCR6$^+$ Th cell subsets, we identified that Th9 cells might be functionally important in anti-DNA$^+$ SLE patients. These cells are derived from naive T cells in the presence of cytokines, thymic stromal lymphopoietin (TSLP), transforming growth factor (TGF)-β and IL-4, and would also require interferon regulatory factor-4 (IRF-4) and PU.1 transcription factors (*Staudt et al., 2010*; *Veldhoen et al., 2008*). In SLE patients, there have been few studies that have explored the role of Th9 cells along with its related cytokine IL-9, and have indicated an increase in Th9 cells and IL-9 serum levels in SLE patients in comparison to healthy controls (*Dantas et al., 2015*; *Ouyang et al., 2013*), along with a positive correlation with SLEDAI (*Ouyang et al., 2013*). Therefore, this indicated that Th9 subpopulation might play a role in SLE. Furthermore, using MRL/lpr mice (an animal model of lupus), it has been demonstrated that Th9 cells and IL-9 levels expand in the spleen and kidney of these mice, and neutralizing IL-9 antibody lead to diminished anti-DNA titers and alleviation of LN (*Yang et al., 2015*). Consistent with these observations, our study also showed that elevated frequencies of Th9 cells showed a positive correlation with ESR in anti-DNA$^+$ SLE patients, while in anti-DNA$^-$ SLE patients, Th9 cells showed no correlation with any of the clinical indicators.

Another CCR6$^+$ Th cell subset which has been shown to be significantly elevated in SLE patients was Th17 cell subset, along with cytokine IL-17, and had been suggested to play a critical role in the development of SLE (*Garrett-Sinha, John & Gaffen, 2008*; *Zhang et al., 2016*). Consistent with this study, we also observed an increase in the levels of Th17 cells in anti-DNA$^+$ SLE patients. This correlation could be accounted for due to the activation of NLRP3 by anti-DNA (*Shin et al., 2013*; *Zhang et al., 2016*), which in turn promotes Th17 cell differentiation (*Bruchard et al., 2013*), and thus contributed to disease severity by expanding pathogenic Th17 cells (*Zhang et al., 2016*). However, due to the high plasticity of Th17 cells, they often shift rapidly into a Th1-like phenotype, resulting in the production of IL-17 and IFN-y tand hence are also referred as Th17/Th1, non-classic Th1

(*Cosmi et al., 2014*), or Th17.1 cells (*Paulissen et al., 2015b*). It has also been shown that Th17.1 cells can be polarized from from naïve T cells directly, in *C. albicans*-primed cultures (*Zielinski et al., 2012*). Th17.1 cells appear to be more pathogenic than Th17 in human inflammatory disorders (*Cosmi et al., 2014*), and express transporter protein multi-drug resistance type 1 (MDR1), which results in insensitivity to glucocorticoids (*Ramesh et al., 2014*). Elevation of Th17.1 cells levels have been observed in anti-citrullinated protein antibodies (ACPA) positive RA patients with worse disease outcome, than in ACPA negative RA patients (*Paulissen et al., 2015b*). Notably, we also observed higher levels of Th17.1 cells and their negative correlation with C3 protein levels in anti-DNA$^+$ SLE patients, thereby suggesting that Th17.1 probably contribute to a higher disease activity and worse treatment outcomes in anti-DNA$^+$ SLE patients.

Interestingly, we also observed increased frequencies of CCR4/CXCR3 DN and CCR6$^+$ T cells in anti-DNA$^+$ SLE patients and a correlation of different clinical parameters with the CCR4/CXCR3 DN and DP CCR6$^+$ T cells. DP cells usually show both Th1 and Th17 characteristics, while DN cells have Th17 cell specific features with expression of RORC and CD161 proteins (*Paulissen et al., 2015a*). In RA patients, these DP and DN CCR6$^+$ Th subsets have been shown to manifest a higher activating effect on synovial fibroblasts than Th1 and naive cells (*Paulissen et al., 2015a*). However, their roles in SLE remain unclear and require further analysis.

In addition, CCR6$^+$ T cells also included several additional cell subsets, like CCR6$^+$ Tregs that might play a role in tissue-protection, and has been reported to be elevated during active SLE (*Schmidt et al., 2017*). Therefore, appearance of CCR6$^+$ T cells might just reflect compensatory/protective immunologic reaction provoked by more severe disease in anti-DNA$^+$ patients. Thus, additional studies are warranted to clarify the actual function of these Tregs, as well as the relevance of CCR6 coexpression in different T cell subsets.

CONCLUSIONS

Overall, our data suggested that CCR6$^+$ Th cells may contribute to disease severity in anti-DNA$^+$ SLE patients, as they were specifically elevated in these patients and showed a correlation with ESR. In addition, our results also led us to propose that CCR6$^+$ Th cells may serve as a prognostic indicator for risk-stratification and may prove a novel therapeutic target for the treatment of SLE. It would, therefore, be interesting to further characterize the dynamic role played by these cells specifically in anti-DNA$^+$ SLE patients, using a larger sample size.

ADDITIONAL INFORMATION AND DECLARATIONS

Competing Interests

The authors declare there are no competing interests.

Author Contributions

- Wei Zhong conceived and designed the experiments, performed the experiments, analyzed the data, contributed reagents/materials/analysis tools, wrote the paper, prepared figures and/or tables, reviewed drafts of the paper.
- Zhenyu Jiang analyzed the data, contributed reagents/materials/analysis tools, wrote the paper, reviewed drafts of the paper.
- Jiang Wu analyzed the data, contributed reagents/materials/analysis tools, reviewed drafts of the paper.
- Yanfang Jiang conceived and designed the experiments, analyzed the data, contributed reagents/materials/analysis tools, reviewed drafts of the paper.
- Ling Zhao conceived and designed the experiments, performed the experiments, analyzed the data, contributed reagents/materials/analysis tools, reviewed drafts of the paper.

Human Ethics

The following information was supplied relating to ethical approvals (i.e., approving body and any reference numbers):

This research was approved by the Human Ethics Committee of Jilin University, China (approval number: 2016-373).

Data Availability

The following information was supplied regarding data availability:

The raw data has been provided as a Supplemental File.

Supplemental Information

Supplemental information for this article can be found online at http://dx.doi.org/10.7717/peerj.4294#supplemental-information.

REFERENCES

Acosta-Rodriguez EV, Rivino L, Geginat J, Jarrossay D, Gattorno M, Lanzavecchia A, Sallusto F, Napolitani G. 2007. Surface phenotype and antigenic specificity of human interleukin 17-producing T helper memory cells. *Nature Immunology* **8**:639–646 DOI 10.1038/ni1467.

Arbuckle MR, McClain MT, Rubertone MV, Scofield RH, Dennis GJ, James JA, Harley JB. 2003. Development of autoantibodies before the clinical onset of

systemic lupus erythematosus. *New England Journal of Medicine* **349**:1526–1533 DOI 10.1056/NEJMoa021933.

Bombardier C, Gladman DD, Urowitz MB, Caron D, Chang CH. 1992. Derivation of the SLEDAI. A disease activity index for lupus patients. The Committee on Prognosis Studies in SLE. *Arthtitis and Rheumatism* **35**:630–640 DOI 10.1002/art.1780350606.

Bonecchi R, Bianchi G, Bordignon PP, D'Ambrosio D, Lang R, Borsatti A, Sozzani S, Allavena P, Gray PA, Mantovani A, Sinigaglia F. 1998. Differential expression of chemokine receptors and chemotactic responsiveness of type 1 T helper cells (Th1s) and Th2s. *Journal of Experimetnal Medicine* **187**:129–134 DOI 10.1084/jem.187.1.129.

Bruchard M, Mignot G, Derangere V, Chalmin F, Chevriaux A, Vegran F, Boireau W, Simon B, Ryffel B, Connat JL, Kanellopoulos J, Martin F, Rebe C, Apetoh L, Ghiringhelli F. 2013. Chemotherapy-triggered cathepsin B release in myeloid-derived suppressor cells activates the Nlrp3 inflammasome and promotes tumor growth. *Nature Medicine* **19**:57–64 DOI 10.1038/nm.2999.

Conti F, Ceccarelli F, Perricone C, Massaro L, Marocchi E, Miranda F, Spinelli FR, Truglia S, Alessandri C, Valesini G. 2015. Systemic lupus erythematosus with and without anti-dsDNA antibodies: analysis from a Large Monocentric Cohort. *Mediators of Inflammation* **2015**:Article 328078 DOI 10.1155/2015/328078.

Cosmi L, Liotta F, Maggi E, Romagnani S, Annunziato F. 2014. Th17 and non-classic Th1 cells in chronic inflammatory disorders: two sides of the same coin. *International Archives of Allergy and Immunology* **164**:171–177 DOI 10.1159/000363502.

Crispin JC, Oukka M, Bayliss G, Cohen RA, Van Beek CA, Stillman IE, Kyttaris VC, Juang YT, Tsokos GC. 2008. Expanded double negative T cells in patients with systemic lupus erythematosus produce IL-17 and infiltrate the kidneys. *Journal of Immunology* **181**:8761–8766 DOI 10.4049/jimmunol.181.12.8761.

Dantas AT, Marques CD, Da Rocha Jr LF, Cavalcanti MB, Goncalves SM, Cardoso PR, Mariz Hde A, Rego MJ, Duarte AL, Pitta Ida R, Pitta MG. 2015. Increased serum interleukin-9 levels in rheumatoid arthritis and systemic lupus erythematosus: pathogenic role or just an epiphenomenon? *Disease Markers* **2015**:Article 519638 DOI 10.1155/2015/519638.

Datta SK. 1998. Production of pathogenic antibodies: cognate interactions between autoimmune T and B cells. *Lupus* **7**:591–596 DOI 10.1191/096120398678920703.

Duhen T, Geiger R, Jarrossay D, Lanzavecchia A, Sallusto F. 2009. Production of interleukin 22 but not interleukin 17 by a subset of human skin-homing memory T cells. *Nature Immunology* **10**:857–863 DOI 10.1038/ni.1767.

Enghard P, Humrich JY, Rudolph B, Rosenberger S, Biesen R, Kuhn A, Manz R, Hiepe F, Radbruch A, Burmester GR, Riemekasten G. 2009. CXCR3+CD4+ T cells are enriched in inflamed kidneys and urine and provide a new biomarker for acute nephritis flares in systemic lupus erythematosus patients. *Arthtitis and Rheumatism* **60**:199–206 DOI 10.1002/art.24136.

Fattal I, Shental N, Molad Y, Gabrielli A, Pokroy-Shapira E, Oren S, Livneh A, Langevitz P, Pauzner R, Sarig O, Gafter U, Domany E, Cohen IR. 2014. Epstein-Barr virus antibodies mark systemic lupus erythematosus and scleroderma patients negative for anti-DNA. *Immunology* **141**:276–285 DOI 10.1111/imm.12200.

Garrett-Sinha LA, John S, Gaffen SL. 2008. IL-17 and the Th17 lineage in systemic lupus erythematosus. *Current Opinion in Rheumatology* **20**:519–525 DOI 10.1097/BOR.0b013e328304b6b5.

Hahn BH, McMahon MA, Wilkinson A, Wallace WD, Daikh DI, Fitzgerald JD, Karpouzas GA, Merrill JT, Wallace DJ, Yazdany J, Ramsey-Goldman R, Singh K, Khalighi M, Choi SI, Gogia M, Kafaja S, Kamgar M, Lau C, Martin WJ, Parikh S, Peng J, Rastogi A, Chen W, Grossman JM, American College of Rheumatology. 2012. American College of Rheumatology guidelines for screening, treatment, and management of lupus nephritis. *Arthritis Care & Research* **64**:797–808 DOI 10.1002/acr.21664.

Hirota K, Yoshitomi H, Hashimoto M, Maeda S, Teradaira S, Sugimoto N, Yamaguchi T, Nomura T, Ito H, Nakamura T, Sakaguchi N, Sakaguchi S. 2007. Preferential recruitment of CCR6-expressing Th17 cells to inflamed joints via CCL20 in rheumatoid arthritis and its animal model. *Journal of Experimental Medicine* **204**:2803–2812 DOI 10.1084/jem.20071397.

Hochberg MC. 1997. Updating the American College of Rheumatology revised criteria for the classification of systemic lupus erythematosus. *Arthtitis and Rheumatism* **40**:1725 DOI 10.1002/1529-0131(199709)40:9<1725::AID-ART29>3.0.CO;2-Y.

Kaul A, Gordon C, Crow MK, Touma Z, Urowitz MB, Van Vollenhoven R, Ruiz-Irastorza G, Hughes G. 2016. Systemic lupus erythematosus. *Nature Reviews Disease Primers* **2**:Article 16039 DOI 10.1038/nrdp.2016.39.

Koga T, Otomo K, Mizui M, Yoshida N, Umeda M, Ichinose K, Kawakami A, Tsokos GC. 2016. Calcium/calmodulin-dependent kinase iv facilitates the recruitment of interleukin-17-producing cells to target organs through the CCR6/CCL20 axis in Th17 cell-driven inflammatory diseases. *Arthritis & Rheumatology* **68**:1981–1988 DOI 10.1002/art.39665.

Lazarus MN, Turner-Stokes T, Chavele KM, Isenberg DA, Ehrenstein MR. 2012. B-cell numbers and phenotype at clinical relapse following rituximab therapy differ in SLE patients according to anti-dsDNA antibody levels. *Rheumatology* **51**:1208–1215 DOI 10.1093/rheumatology/ker526.

Linnik MD, Hu JZ, Heilbrunn KR, Strand V, Hurley FL, Joh T, LJP 394 Investigator Consortium. 2005. Relationship between anti-double-stranded DNA antibodies and exacerbation of renal disease in patients with systemic lupus erythematosus. *Arthtitis and Rheumatism* **52**:1129–1137 DOI 10.1002/art.20980.

Mahnke YD, Beddall MH, Roederer M. 2013. OMIP-017: human CD4(+) helper T-cell subsets including follicular helper cells. *Cytometry A* **83**:439–440 DOI 10.1002/cyto.a.22269.

Mavragani CP, Fragoulis GE, Somarakis G, Drosos A, Tzioufas AG, Moutsopoulos HM. 2015. Clinical and laboratory predictors of distinct histopathogical features of lupus nephritis. *Medicine* **94**:e829 DOI 10.1097/MD.0000000000000829.

Mohan C, Adams S, Stanik V, Datta SK. 1993. Nucleosome: a major immunogen for pathogenic autoantibody-inducing T cells of lupus. *Journal of Experimental Medicine* **177**:1367–1381 DOI 10.1084/jem.177.5.1367.

Ouyang H, Shi YB, Su N, Li LY. 2013. Abnormality and significance of interleukin-9 and CD4(+)interleukin-9(+) T-cells in peripheral blood of patients with systemic lupus erythematosus. *Zhonghua Yi Xue Za Zhi* **93**:99–103.

Paulissen SM, Van Hamburg JP, Dankers W, Lubberts E. 2015a. The role and modulation of CCR6+ Th17 cell populations in rheumatoid arthritis. *Cytokine* **74**:43–53 DOI 10.1016/j.cyto.2015.02.002.

Paulissen SM, Van Hamburg JP, Davelaar N, Vroman H, Hazes JM, De Jong PH, Lubberts E. 2015b. CCR6(+) Th cell populations distinguish ACPA positive from ACPA negative rheumatoid arthritis. *Arthritis Research & Therapy* **17**:Article 344 DOI 10.1186/s13075-015-0800-5.

Pisetsky DS. 2016. Anti-DNA antibodies–quintessential biomarkers of SLE. *Nature Reviews Rheumatology* **12**:102–110 DOI 10.1038/nrrheum.2015.151.

Podrebarac TA, Boisert DM, Goldstein R. 1998. Clinical correlates, serum autoantibodies and the role of the major histocompatibility complex in French Canadian and non-French Canadian Caucasians with SLE. *Lupus* **7**:183–191 DOI 10.1191/096120398678919976.

Ramesh R, Kozhaya L, McKevitt K, Djuretic IM, Carlson TJ, Quintero MA, McCauley JL, Abreu MT, Unutmaz D, Sundrud MS. 2014. Pro-inflammatory human Th17 cells selectively express P-glycoprotein and are refractory to glucocorticoids. *Journal of Experimetnal Medicine* **211**:89–104 DOI 10.1084/jem.20130301.

Ray SK, Putterman C, Diamond B. 1996. Pathogenic autoantibodies are routinely generated during the response to foreign antigen: a paradigm for autoimmune disease. *Proceedings of the National Academy of Sciences of the United States of America* **93**:2019–2024 DOI 10.1073/pnas.93.5.2019.

Rivino L, Messi M, Jarrossay D, Lanzavecchia A, Sallusto F, Geginat J. 2004. Chemokine receptor expression identifies Pre-T helper (Th)1, Pre-Th2, and nonpolarized cells among human CD4+ central memory T cells. *Journal of Experimetnal Medicine* **200**:725–735 DOI 10.1084/jem.20040774.

Robak E, Niewiadomska H, Robak T, Bartkowiak J, Blonski JZ, Wozniacka A, Pomorski L, Sysa-Jedrezejowska A. 2001. Lymphocyctes Tgammadelta in clinically normal skin and peripheral blood of patients with systemic lupus erythematosus and their correlation with disease activity. *Mediators of Inflammation* **10**:179–189 DOI 10.1080/09629350124724.

Sallusto F, Mackay CR, Lanzavecchia A. 2000. The role of chemokine receptors in primary, effector, and memory immune responses. *Annual Review of Immunology* **18**:593–620 DOI 10.1146/annurev.immunol.18.1.593.

Schiffenbauer J, Simon LS. 2004. Randomized controlled trials in systemic lupus erythematosus: what has been done and what do we need to do? *Lupus* **13**:398–405 DOI 10.1191/0961203303lu1033oa.

Schmidt A, Rieger CC, Venigalla RK, Elias S, Max R, Lorenz HM, Grone HJ, Krammer PH, Kuhn A. 2017. Analysis of FOXP3(+) regulatory T cell subpopulations in peripheral blood and tissue of patients with systemic lupus erythematosus. *Immunologic Research* **65**:551–563 DOI 10.1007/s12026-017-8904-4.

Sherer Y, Gorstein A, Fritzler MJ, Shoenfeld Y. 2004. Autoantibody explosion in systemic lupus erythematosus: more than 100 different antibodies found in SLE patients. *Seminars in Arthritis and Rheumatism* **34**:501–537 DOI 10.1016/j.semarthrit.2004.07.002.

Shin MS, Kang Y, Lee N, Wahl ER, Kim SH, Kang KS, Lazova R, Kang I. 2013. Self double-stranded (ds)DNA induces IL-1beta production from human monocytes by activating NLRP3 inflammasome in the presence of anti-dsDNA antibodies. *Journal of Immunology* **190**:1407–1415 DOI 10.4049/jimmunol.1201195.

Staudt V, Bothur E, Klein M, Lingnau K, Reuter S, Grebe N, Gerlitzki B, Hoffmann M, Ulges A, Taube C, Dehzad N, Becker M, Stassen M, Steinborn A, Lohoff M, Schild H, Schmitt E, Bopp T. 2010. Interferon-regulatory factor 4 is essential for the developmental program of T helper 9 cells. *Immunity* **33**:192–202 DOI 10.1016/j.immuni.2010.07.014.

Tan EM, Cohen AS, Fries JF, Masi AT, McShane DJ, Rothfield NF, Schaller JG, Talal N, Winchester RJ. 1982. The 1982 revised criteria for the classification of systemic lupus erythematosus. *Arthtitis and Rheumatism* **25**:1271–1277 DOI 10.1002/art.1780251101.

Trifari S, Kaplan CD, Tran EH, Crellin NK, Spits H. 2009. Identification of a human helper T cell population that has abundant production of interleukin 22 and is distinct from T(H)-17, T(H)1 and T(H)2 cells. *Nature Immunology* **10**:864–871 DOI 10.1038/ni.1770.

Turner JE, Paust HJ, Steinmetz OM, Peters A, Riedel JH, Erhardt A, Wegscheid C, Velden J, Fehr S, Mittrucker HW, Tiegs G, Stahl RA, Panzer U. 2010. CCR6 recruits regulatory T cells and Th17 cells to the kidney in glomerulonephritis. *Journal of the American Society of Nephrology* **21**:974–985 DOI 10.1681/ASN.2009070741.

Van Hamburg JP, Corneth OB, Paulissen SM, Davelaar N, Asmawidjaja PS, Mus AM, Lubberts E. 2013. IL-17/Th17 mediated synovial inflammation is IL-22 independent. *Annals of the Rheumatic Diseases* **72**:1700–1707 DOI 10.1136/annrheumdis-2012-202373.

Veldhoen M, Uyttenhove C, Van Snick J, Helmby H, Westendorf A, Buer J, Martin B, Wilhelm C, Stockinger B. 2008. Transforming growth factor-beta 'reprograms' the differentiation of T helper 2 cells and promotes an interleukin 9-producing subset. *Nature Immunology* **9**:1341–1346 DOI 10.1038/ni.1659.

Vincent FB, Northcott M, Hoi A, Mackay F, Morand EF. 2013. Clinical associations of serum interleukin-17 in systemic lupus erythematosus. *Arthritis Research & Therapy* **15**:Article R97 DOI 10.1186/ar4277.

Yang J, Li Q, Yang X, Li M. 2015. Interleukin-9 is associated with elevated anti-double-stranded DNA antibodies in lupus-prone mice. *Molecular Medicine* **21**:364–370 DOI 10.2119/molmed.2014.00237.

Ye ZJ, Yuan ML, Zhou Q, Du RH, Yang WB, Xiong XZ, Zhang JC, Wu C, Qin SM, Shi HZ. 2012. Differentiation and recruitment of Th9 cells stimulated by pleural mesothelial cells in human Mycobacterium tuberculosis infection. *PLOS ONE* **7**:e31710 DOI 10.1371/journal.pone.0031710.

Zhang H, Fu R, Guo C, Huang Y, Wang H, Wang S, Zhao J, Yang N. 2016. Anti-dsDNA antibodies bind to TLR4 and activate NLRP3 inflammasome in lupus monocytes/macrophages. *Journal of Translational Medicine* **14**:Article 156 DOI 10.1186/s12967-016-0911-z.

Zhao L, Jiang Z, Jiang Y, Ma N, Wang K, Zhang Y, Feng L. 2013. IL-22+CD4+ T-cells in patients with active systemic lupus erythematosus. *Experimental Biology and Medicine* **238**:193–199 DOI 10.1177/1535370213477597.

Zhou XJ, Mu R, Li C, Nath SK, Zhang YM, Qi YY, Li ZG, Zhao MH, Zhang H. 2015. Association of variants in CCR6 with susceptibility to lupus nephritis in Chinese. *Arthritis & Rheumatology* **67**:3091–3093 DOI 10.1002/art.39268.

Zielinski CE, Mele F, Aschenbrenner D, Jarrossay D, Ronchi F, Gattorno M, Monticelli S, Lanzavecchia A, Sallusto F. 2012. Pathogen-induced human TH17 cells produce IFN-gamma or IL-10 and are regulated by IL-1beta. *Nature* **484**:514–518 DOI 10.1038/nature10957.