ORIGINAL ARTICLE

Analysis of CD95 and CCR7 expression on circulating CD4$^+$ lymphocytes revealed disparate immunoregulatory potentials in systemic lupus erythematosus

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Abstract Emerging data have implicated a critical role for CD4 in the pathogenesis of systemic lupus erythematosus (SLE). This study was designed to delineate the contribution of CD4$^+$ T cells in the pathogenesis of SLE disease. Forty-four patients (3 male: 41 female) and 20 healthy volunteers (4 male: 16 female) were included in the study. CD4$^+$ lymphocytes analysis was done using three-color flow cytometry with antibodies against human-CD95, a prototype cell death receptor, and the chemokine receptor-7 (CCR7) after gating for lymphocytes based on the forward and side scatter. Serum levels of IL-6, IL-12, IL-17, TNF-α and IL-10 cytokines were assayed using ELISA. Disease activity was assessed using the SLE disease activity index (SLEDAI). Based on the expression of CCR7 and CD95, CD4$^+$ lymphocytes were subdivided into three particular subsets; CD4$^+$CD95$^+$CCR7$^+$ cells, CD4$^+$CD95$^-$CCR7$^+$ cells and CD4$^+$CD95$^+$CCR7$^-$ cells. Percentage of CD4$^+$CD95$^+$CCR7$^+$ cell subset was significantly higher in patients with SLE with

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active disease (SLEDAI > 6) and inactive (SLEDAI < 6) as compared with controls ($P = 0.005$), and it showed a significant positive correlation with ANA titer ($P = 0.01$), and a negative correlation with WBCs count ($P = 0.001$). $CD4^+CD95^+CCR7^-$ cell subset was significantly higher in active SLE patients in comparison to patients with inactive disease and controls ($P = 0.05$, $P = 0.005$ respectively), and it correlates positively with SLEDAI, IL-6 and IL-17 levels ($P = 0.001$, 0.05, 0.01 respectively), and negatively with blood WBCs counts ($P = 0.001$). The third $CD4^+CD95^-CCR7^+$ cell subset was found significantly lower in SLE patients compared with controls, and it was found negatively correlated with IL-10, IL-6, and IL-17. The results show that $CD4^+CD95^+$ subset lacking expression of CCR7 is associated with cell mediated inflammatory response as manifested by its correlation with signs of inflammation, inflammatory cytokines and disease activity index. Whereas, $CD4^+CD95^+CCR7^+$ correlate more with antibody immune responses as manifested by association with serum ANA. These data suggest disparate roles of these cell subsets in the pathophysiology of SLE. A better understanding of the characteristics of CD4 cell subsets may shed light on the pathogenesis of autoimmune diseases, particularly SLE.

1. Introduction

Systemic lupus erythematosus (SLE) is one of the most common autoimmune diseases that can affect multiple organs and body systems. The etiology of the disease remains unclear; however, it is thought to be triggered by autoantigens that induce autoantibody production. Apoptotic lymphocytes may be one source of autoantigens, and increased numbers of apoptotic lymphocytes have been reported in SLE (Shah et al., 2011). Notably, apoptotic $CD4^+$, but not $CD8^+$, T lymphocytes were found to constitute the main source for the activated lymphocyte derived DNA and other apoptotic cellular constituents that function as autoantigens to induce production of anti-double-stranded DNA (anti-dsDNA) antibodies in SLE (Wen et al., 2012).

Several studies reported increased levels of apoptotic $CD4^+$ lymphocytes in SLE patients; these lymphocytes are characterized by the expression of CD95, (Fas/APO-1), the prototypic death receptor of the immune system (Bijl et al., 2001; Xue et al., 2006). CD95 is a member of the TNF receptor superfamily, whose ligand, CD95L (FasL), is expressed on activated; but not resting T cells (Garrone et al., 1995; Genestier et al., 1998). In addition to its role in apoptosis, CD95 binding to its agonist was found to activate the nuclear factor-kappa B (NF-κB), a transcriptional factor that plays critical roles in inflammation, immunity, cell proliferation, differentiation, and survival (Rensing-Ehl et al., 1995). NF-κB is involved in the transcriptional regulation of many cytokine (Rensing-Ehl et al., 1995), and chemokine (Liu et al., 2004; Tang et al., 2009) genes expressed in activated lymphocytes. Thus, in addition to provoking apoptosis, CD95 receptor stimulation can indirectly trigger inflammation and lymphocyte migration to lymph nodes or inflamed peripheral tissues (Cullen et al., 2013).

In essence, the interaction between T-B lymphocytes occurring in lymphoid tissues requires the migration of T and B-cells to their specified T- and B-cell zones in lymphoid tissue where they encounter dendritic cells and antigens (Eriksson and Rantapaa-Dahlqvist, 2014). This process depends on migration of $CD4^+$ T cell to secondary lymphoid organs and the multitude of autoantigens that can provide. Previous investigations show that $CD4^+$ T cells expressing the chemokine receptor-7 (CCR7), which recognizes CCL19 and CCL21, are attracted close to B cell follicles where it triggers the activation and differentiation (T-cell help) of B cells in germinal centers of the lymphoid tissue (MacLennan, 1994; Okada and Cyster, 2006).

The aforementioned data suggest that the surface receptor CD95 is involved in other physiological functions besides apoptotic signaling, such as inflammation cytokine and/or chemokine receptor expression. Therefore, we aimed to investigate the extent of $CD4^+$ lymphocytes expressing CD95 and their association with chemokine receptor CCR7 expression, inflammatory cytokines and disease activity in patients with SLE.

2. Materials and methods

2.1. Patients

A total of 44 SLE patients were classified according to the SLE disease activity index (SLEDAI) (Bombardier et al., 1992), into active group defined as SLEDAI score > 6 (included 3 males and 23 Females) patients, and inactive group defined as SLEDAI score less than 6 (included 18 females). Twenty healthy volunteers (4 males and 16 females), who were clinically and laboratory free of any autoimmune diseases, served as a control group. All patients have provided written informed consent for study inclusion and the anonymous use of their data from lupus disease registry. The King Abdulaziz University Hospital Ethics Committee has approved the study.

2.2. Clinical and laboratory assessments

Clinical and demographic characteristics were recorded for all patients shown in (Table 1). Levels of anti-nuclear antibodies (ANA), anti-dsDNA antibody, C-reactive protein (CRP), erythrocyte sedimentation rate (ESR), complete blood count (CBC), complement (C)-3, C4, urine analysis were assayed using commercially available kits according to the manufacturer's instructions.

2.3. Flow cytometry analysis

Peripheral blood mononuclear cells (PBMCs) were analyzed by three-color flow cytometry using CD95-FITC, CCR7-PE, and

Table 1 Demographic, clinical and laboratory data for the studied groups.

Parameters	Active SLE ($n = 26$)	Inactive SLE ($n = 18$)	HCs ($n = 20$)
Demographic and clinical data			
Gender (male/female)	3/23	0/18	4/16
Age (years)	35.44 ± 11.36	31.81 ± 10.21	29.15 ± 4.74
Disease duration (years)	5.88 ± 3.99	4.86 ± 1.43	
SLEDAI	15.57 ± 7.69	3.5 ± 2	
Laboratory data			
IL-6 (ng/ml)	9.55 ± 2.23[a]	9.72 ± 1.91[a]	3.47 ± 1.41
IL-12 (ng/ml)	21.59 ± 1.66[a]	22.71 ± 0.67[a]	15.42 ± 1.16
TNF-α (ng/ml)	19.49 ± 2.18[a,b]	16.83 ± 1.54[a]	8.99 ± 0.47
IL-17 (ng/ml)	41.65 ± 2.13[a,b]	33.53 ± 2.11[a]	27.47 ± 1.29
IL-10 (ng/ml)	21.9 ± 2.43[a]	19.27 ± 2.35[a]	13.83 ± 1.35
ANA (IU/ml)	0.75 ± 0.33[a]	0.53 ± 0.39 [a]	0.11 ± 0.04
Anti ds-DNA (IU/ml)	665.85 ± 401.2[b]	445.52 ± 217.44	Neg.
C3 (g/L) (0.75–1.65)	0.84 ± 0.32[a]	0.93 ± 0.34	0.78 ± 0.14
C4 (g/L) (0.2–0.6)	0.14 ± 0.43[a]	0.16 ± 0.09	0.29 ± 0.07
CRP (mg/L)	4.85 ± 2.16[a]	4.99 ± 3.57[a]	2.46 ± 0.19
ESR (mm/H)	25.64 ± 16.25[a]	26.81 ± 13.21[a]	11.8 ± 6.25
WBC (K/UL)	3.35 ± 1.58[a,b]	5.57 ± 1.26	8.64 ± 2.99
RBC (M/UL)	4.50 ± 0.69	4.27 ± 0.72	4.52 ± 0.47
PLT (K/UL)	279.07 ± 68.16	243.13 ± 67.05	268.4 ± 71.02
WBC in urine	9.43 ± 6.13[b]	2.24 ± 2.41	–
RBC in urine	8.71 ± 7.87[b]	2.24 ± 2.12	–

Values represent mean ± S.D. of means. Superscript letter "a" refers to significant difference in comparison with control group, and "b" with inactive SLE group. The difference is significant at $P < 0.05$ levels.

CD4-Percp. Isotype-matched control mAbs were used to determine the nonspecific binding. All monoclonal antibodies were purchased from R&D system (Minneapolis MN, USA). Flow cytometry analysis was performed on BD FACSCalibur™ flow cytometer and 10,000 events were acquired using Cell Quest Software (BD Biosciences, San Jose, CA, USA). Data were analyzed with the Summit v4.3 Build 2445 software (Dako Colorado Inc., Fort Collins, CO, USA). Percentages of apoptotic CD4 lymphocytes were determined after gating on the forward scatter and side scatter properties of lymphocytes and the CD4-Percp positive population (Fig. 1).

2.4. Cytokine measurement

Serum concentrations of pro-inflammatory (IL-6, IL-12, IL-17 and TNF-α) and anti-inflammatory (IL-10) cytokines were assayed by using commercial ELISA kits (ELISA MAX™ Deluxe Sets, BioLegened; San Diego CA, USA). All measurements were performed in duplicate according to the manufacture's recommendations.

2.5. Statistical analysis

All data were statistically analyzed using Statistical Package for Social Sciences (SPSS) V20.0. The data were expressed as means ± standard deviation (SD). Correlations were done using Pearson tests, and analyses of variance were done using ANOVA test followed by Bonferroni correction. P-value less than or equal to 0.05 is considered as being statistically significant.

3. Results

Table 1 presents the demographic, clinical and laboratory data for the studied groups. It is apparent that there is a significant difference ($P = 0.0001$) in SLEDAI between active and inactive groups, whereas, the differences in age and number of abortions and pregnancies (in female patients) and disease duration did not show statistical significance.

The concentrations of TNF-a and IL-17 were significantly higher in the active SLE group than that of inactive SLE ($P = 0.05$, $P = 0.02$ respectively) and healthy ($P = 0.01$, $P = 0.001$ respectively) subjects. Whereas the concentrations of IL-6, IL-10 and IL-12 cytokines were significantly higher in both active and inactive SLE patients compared with healthy controls (Table 1).

Expression of CD95 and CCR7 was analyzed on CD4$^+$ T cells that yield three subsets of CD4 T-cells (Fig. 1). The first subset consists of CD4$^+$CD95$^+$CCR7$^+$ cells that were found significantly higher in active and inactive SLE patients than the healthy control group ($43 ± 2.7\%$ and $42 ± 2.2$ vs. $13 ± 1.4$, $P < 0.0001$). The second cell population includes CD4$^+$CD95$^+$CCR7$^-$ cells which were significantly higher in active ($29 ± 7.2\%$) than inactive ($21.9 ± 5.6\%$) and healthy subjects ($18 ± 4.8\%$) ($P = 0.05$ and 0.01 respectively). The third CD4$^+$CD95$^-$CCR7$^+$ T cell population was significantly higher in control subjects than that in the active SLE group and the inactive SLE group ($49 ± 5.1\%$ vs. $28 ± 13.2\%$ and $31 ± 11.45$), (both at $P < 0.05$) (Fig. 1).

The correlations of each CD4$^+$ T cell subset with other clinical and biochemical parameters are presented in Table 2. CD4$^+$CD95$^+$CCR7$^+$ cells showed a positive correlation with IL-10 and ANA ($r = 0.499$, $P = 0.01$; $r = 0.757$, $P = 0.001$ respectively), and a negative correlation with blood WBCs count ($r = -0.462$, $P = 0.05$). CD4$^+$CD95$^+$CCR7$^-$ cells correlated positively with number of abortions, SLEDAI, IL-6 and IL-17 concentrations ($r = 0.465$, $P = 0.02$; $r = 0.608$, $P = 0.001$; $r = 0.595$, $P = 0.01$ and $r = 0.770$, $P = 0.001$ respectively). Whereas, CD4$^+$CD95$^-$CCR7$^+$ cells correlated
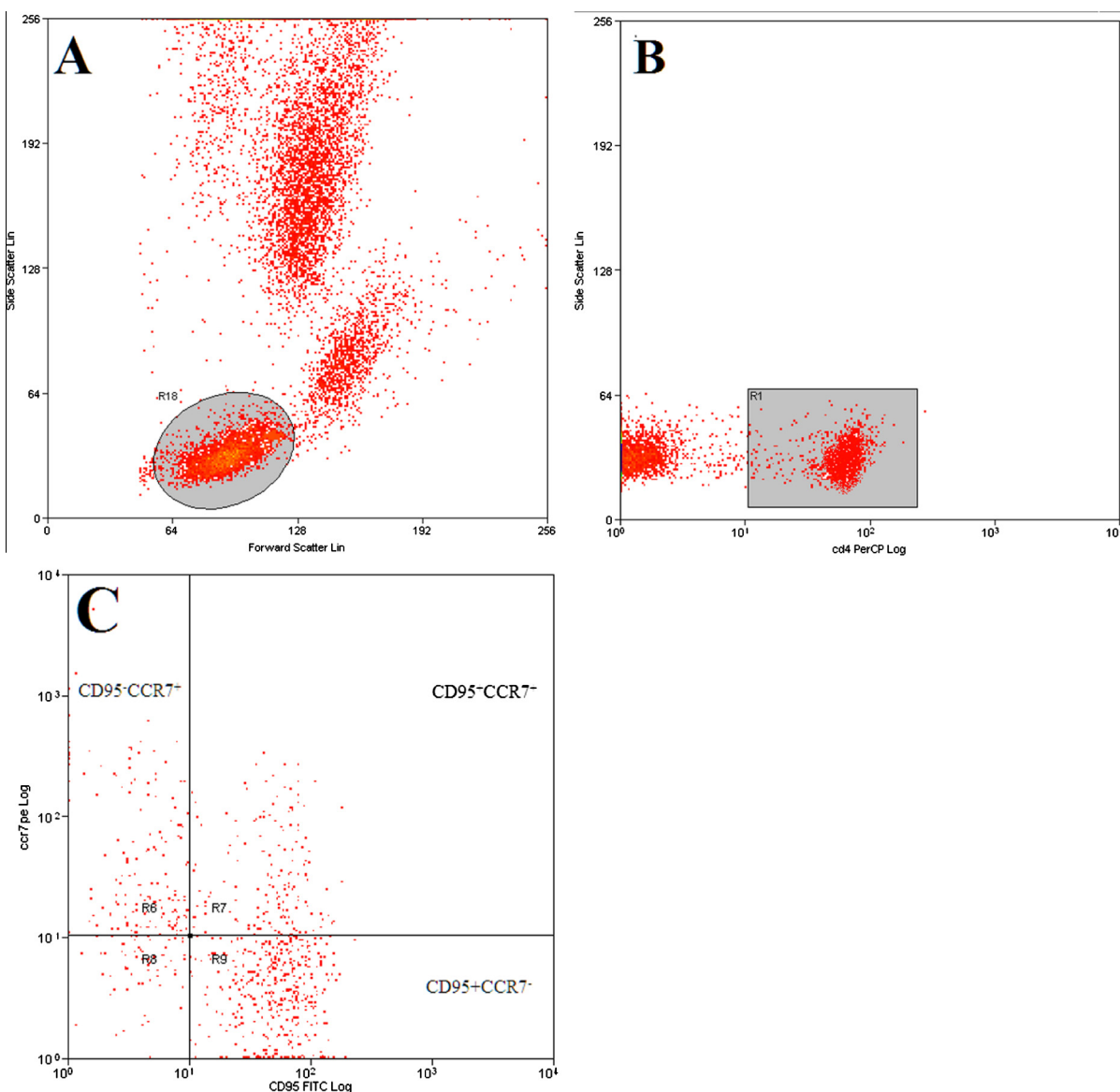
Figure 1 (A–C) Example of the gating strategy used to identify CD4$^+$ T cell-subsets according to expressing of CD95 and CCR7. Gates were set around lymphocytes, as defined by forward and side light scatter (A), and then gating on CD4$^+$ bright positive cells (B). The two gates were applied to the lower dot plot (C) displaying CCR7 and CD95.

negatively with IL-10, IL-17 and TNF-a concentrations ($r = -0.595$, $P = 0.01$; $r = -0.587$, $P = 0.02$; $r = -0.410$, $P = 0.05$, respectively, (Table 2).

4. Discussion

The clinical course of SLE disease is heterogeneous with alternating flares periods of increased disease activity and remissions, when pain and inflammation disappear. The reasons behind this fluctuation in disease activity are unclear; however evidence suggests that apoptotic lymphocytes likely account for this phenomenon. As apoptotic lymphocytes, particularly CD4$^+$ lymphocytes, were found to serve as a source for autoantigens and increase the production of ANA and anti-dsDNA antibodies that are considered hallmarks of SLE (Wen et al., 2012).

In the present study, we found that SLE patients had an increased percentage of CD4$^+$ lymphocytes expressing CD95, the prototype marker of apoptosis, indicating an increased apoptosis of these cells in SLE patients *in vivo*. This finding confirms earlier observations of Wen et al. (2012) that CD95 is upregulated upon activation in SLE. Other studies reported that T cells with increased CD95 antigen expression might be highly susceptible to apoptotic cell death *in vivo* (Amasaki et al., 1995; Silvestris et al., 2002).

Cell death of CD4 lymphocytes by apoptosis represents a putative mechanism for providing an excessive load of apoptotic particles containing nuclear antigens or of immune complexes containing autoantigens that can overcome self-tolerance mechanisms and trigger autoimmunity (Gabler et al., 2003). However, this seems to depend on two processes, CD4$^+$ T cell activation and migration to secondary lymphoid

Table 2 Correlations between CD4$^+$ T cell subsets and clinical parameters in studied groups.

Parameter	CD4$^+$ cell subsets		
	CD4$^+$CD95$^+$CCR7$^+$	CD4$^+$CD95$^+$CCR7$^-$	CD4$^+$CD95$^-$CCR7$^+$
Demographic & clinical data			
Age	0.192	−0.089	−0.302
No. pregnancy	−0.063	0.224	−0.402
No. Of abortion	−0.306	0.456[*]	−0.013
Disease duration	−0.001	0.040	−0.221
SLEDAI	−0.244	0.608[**]	−0.256
Laboratory data			
IL-6	−0.233	0.595[*]	−0.164
IL-10	0.499[*]	−0.127	−0.595[*]
IL-12	0.383	0.164	−0.131
IL-17	−0.304	0.770[**]	−0.587[*]
TNF-alpha	0.341	0.341	−0.410[*]
ANA	0.757[**]	0.302	−0.123
dsDNA	0.246	0.260	0.047
C3	0.008	0.203	−0.097
C4	0.172	−0.119	−0.125
CR-P	0.027	0.097	−0.011
ESR	0.023	0.346	−0.253
WBCs	−0.490[*]	−0.124	0.270
RBs	−0.359	0.201	−0.403
PLT	−0.183	0.099	−0.190
WBC (urine)	−0.327	0.182	−0.230
RBC (urine)	0.054	0.012	−0.092
Total protein	−0.329	0 .258	0.123

[**] Correlation is significant at the 0.01 level (2-tailed).
[*] Correlation is significant at the 0.05 level (2-tailed).

organs where they can interact with B-cells and dendritic cells. Previous investigations showed that T-helper lymphocytes expressing the chemokine receptor CCR7, which recognizes CCL19 and CCL21 chemokines, are attracted close to B cell follicles where it triggers the activation and differentiation of B cells in germinal centers of lymphoid tissue (Okada and Cyster, 2006; Sallusto et al., 2000).

In our study, the expression of CCR7 and CD95 on CD4$^+$ lymphocytes identified three particular subsets that are CD4$^+$CD95$^+$CCR7$^+$ cells, CD4$^+$CD95$^-$CCR7$^+$ cells and CD4$^+$CD95$^+$CCR7$^-$ cells. CD4$^+$CD95$^+$CCR7$^+$ cells-subset was found significantly increased in the blood of both active and inactive SLE patients in comparison to healthy individuals. This cell subset showed a significant positive correlation only with serum IL-10 cells and ANA titer ($P = 0.04$ and 0.004 respectively). IL-10 is a cytokine mainly produced by B cells, which use it to support their activation and antibody production (Tyrrell-Price et al., 2001; Xu et al., 2004). This may explain our finding of the concomitant positive correlation between IL-10 and ANA titer and their association with SLEDAI.

In their study of the different types of CD4$^+$cell subsets; Sallusto et al. (1999) found that a subset of CD4$^+$ cells that express CCR7$^+$ home to the T cell areas of lymphoid organs where they readily proliferate and differentiate into effector cells, marked by the downregulation of CCR7. Recently, this subset of effector CD4$^+$CCR7$^-$ T cells was found migrate to inflamed tissues and display immediate effector functions (Sallusto et al., 2014). In addition, it was found that lack of CCR7 associated with the manifestation of spontaneous autoimmunity (Forster et al., 2008). Furthermore, Kuwabara

et al. (2009) demonstrated that a large proportion of CCR7$^-$ cells is Th17 population that home to inflamed tissues and secretes inflammatory cytokines mainly IL-17, IL-6 and TNF-α in autoimmune encephalomyelitis. In line with these data, our results showed that CD4$^+$CD95$^+$CCR7$^-$ T cells correlated positively with signs of inflammation including number of recurrent spontaneous abortions in female patients and SLEDAI ($P < 0.033$ and 0.005 respectively). In addition, this cell subset showed significant positive correlations with pro-inflammatory cytokines IL-6 and IL-17 ($P < 0.012$ and 0.003 respectively).

IL-17 is an essential cytokine in the pathogenesis of SLE, particularly in the development of tissue damage (Apostolidis et al., 2011). Increased production IL-17 in patients with SLE was found to amplify the immune response by augmenting the production of antibodies by B cells (Crispin and Tsokos, 2010). Th17 cells were found increasing target-organ inflammation by producing several kinds of inflammatory cytokines in addition to IL-17, including TNF-α and IL-6 that may exacerbate SLE disease activity (Rana et al., 2012). The positive correlation between CD4$^+$CD95$^+$ CCR7$^-$ cells and IL-17 in the present study may explain their association with various clinical manifestations of inflammation, and disease activity as manifested by SLEDAI.

The third subset of cells we found is CD4$^+$CD95$^-$CCR7$^+$ lymphocytes, which showed a significant higher percentage in healthy subjects, compared with SLE groups. This subset of cells showed a significant negative correlation with IL-10 ($P < 0.026$), IL-17 ($P < 0.012$), and TNF-α ($P < 0.029$), that may suggest their involvement in anti-inflammatory response. These data are in line with previous studies which reported

that higher levels of naive and lower levels of memory CD4$^+$ T-cells are associated with a more anti-inflammatory profile (Gordon et al., 1996; Ugarte-Gil et al., 2014). The persistence of autoreactive memory cells is thought to be involved in the repeated cycles of remission and recurrent inflammation in SLE (Alecsandru et al., 2011; Leiva et al., 2013).

In conclusion, the present results suggest profound differences between the two CD4$^+$ cell subsets according to the expression of CD95 and CCR7. One subset CD4$^+$CD95$^+$CCR7$^+$ might have a role inactivation of humoral immune response and increasing ANA titer in one hand, whereas CD4$^+$CD95$^+$CCR7$^-$ cells subset seem to be involved in inflammatory response and disease activity as indicated by association with inflammatory cytokines IL-17 and IL-6. Taken together, our results suggest that both CD4$^+$CD95$^+$ cell subsets expressing and lacking the expression of CCR7 have variable roles in the pathophysiology of SLE. A better understanding of the characteristics of these cells may shed light on the pathogenesis of autoimmune diseases, particularly SLE.

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