Increased IL-23R+ Th Cells Population Exhibits Higher SLEDAI-2K Scores in Systemic Lupus Erythematosus Patients

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The IL-23/IL-17 axis plays causative roles in the development and progression of systemic lupus erythematosus (SLE). However, it remains unclear if the IL-17RA+ and IL-23R+ T helper (Th) cells populations are associated with the serum IL-17 and IL-23 levels, or with the immunological parameters and disease activities in SLE patients. Herein, we examined the proportion of IL-17RA+ and IL-23R+ Th cells and serum levels of IL-17 and IL-23 in established SLE patients (n = 50) compared with healthy controls (n = 50). The associations of these interleukins and their receptors with immunological parameters [anti-nuclear antibody (ANA), anti-dsDNA antibody, and C-reactive protein (CRP)] and SLE disease activity (SLEDAI-2K scores) in SLE patients were assessed. $CD3^+CD4^+$ Th cells of SLE patients demonstrated significantly elevated IL-17RA+ ($p = 1.12 \times 10^{-4}$) or IL-23R+ ($p = 1.98 \times 10^{-29}$) populations compared with the healthy controls. Serum IL-17 levels were significantly lower in SLE patients compared with the healthy controls ($p = 8.32 \times 10^{-5}$), while no significant difference was observed for the IL-23 serum levels between both groups. IL-23R+ Th cells population was significantly associated with higher SLEDAI-2K scores ($p = 0.017$). In multivariate analysis, the proportion of IL-23R+ Th cells remained significantly associated with higher SLEDAI-2K scores independent of prednisolone intake ($p = 0.027$). No associations were observed between the interleukin parameters (i.e., IL-17, IL-23, IL-17RA+ Th cells, and IL-23R+ Th cells) with ANA, anti-dsDNA, and CRP status, suggesting that the IL-17/IL-23 axis acts independently of these immunological parameters. In conclusion, our results support that therapeutic inhibition of the IL-23/IL-17 axis receptors on Th cells, particularly IL-23R, is potentially relevant in SLE patients.

Keywords: systemic lupus erythematosus, IL-23/IL-17 axis, IL-17, IL-23, IL-17RA, IL-23R, SLEDAI-2K

INTRODUCTION

Interleukin (IL) is a group of cytokines crucial in regulating immune responses such as inflammation as well as mediating immune cells growth, differentiation, activation, and migration. Imbalanced T cell subsets have been identified in SLE patients associated with SLE immunopathogenesis (1–5). Naïve CD4+ T helper (Th) cells differentiate on activation into various

cell subsets, namely Th1, Th2, and Th17, with specific profile of cytokines production and different immunopathological implications. IL-17 produced by Th17 cells has been implicated in the pathogenesis of autoimmune diseases including SLE (4). IL-23 is produced by macrophages and dendritic cells to promote the expansion and survival of Th17 cells for the production of IL-17 (6). By stimulating pro-inflammatory cytokines production, IL-23 is able to activate memory T cells, affect IFN-γ production, and trigger the response of Th1 cells, which plays crucial roles in the development of SLE (7–10). IL-23 can aggravate autoimmune severity by stimulating the activities of pathogenic Th17 cells to continually produce IL-17 (11), and this pathogenic pathway is termed as the IL-23/IL-17 axis in SLE (12, 13).

It is thought that cytokine receptors identification is more therapeutically beneficial on account of its stability as well as cytokines pleomorphism. Cytokine receptors activate intracellular signaling which controls a wide range of biological and clinically relevant functions including inflammatory reactions and the expansion of immune cells. The receptor for IL-17 cytokine, IL-17RA, has a cytoplasmic tail with docking sites for various signaling intermediates and has been recognized at elevated levels in specific tissues such as the thymus, bone marrow, spleen tissues, and hematopoietic cells (14). IL-23R, a receptor for IL-23, is expressed by various immune cells including dendritic cells, macrophages, and T cells (15). IL-23 and its receptor IL-23R are necessary for the proliferation of pathogenic IL-17-producing T cells shown to be critical for lupus pathogenesis (16), and IL-23R-deficit prevents the development of lupus nephritis in lupus-prone mice (16).

Multiple studies have reported that elevated IL-17 serum levels in SLE patients is associated with higher SLEDAI scores, a parameter to assess SLE disease activities (17–20), compared with the controls (21–26). Independent reports have indicated that Th17 cells can promote the development of SLE (22, 27). Although the initial steps of IL-17 differentiation from naïve CD4⁺ Th cells does not involve IL-23, it has been shown that IL-23 plays a significant role in the expansion and maintenance of Th17 cells, enabling IL-17 to be produced (7, 28–30). Collectively, the IL-23/IL-17 axis plays key roles in the pathogenesis of SLE. However, the profile of IL-17RA⁺ or IL-23R⁺ Th cells in SLE patients compared with healthy individuals, as well as their associations with relevant parameters in the disease, remain unclear.

In this study, we set out to investigate whether serum IL-17 and IL-23 levels or the proportion of IL-17RA⁺ or IL-23R⁺ Th cells differ between SLE patients and the healthy controls. More importantly, the potential associations between these serum cytokines and Th cell subsets with SLE disease activity measured by the SLEDAI-2K scores were examined. Associations of the SLEDAI-2K scores with the clinico-demographical (e.g., age) and immunological (e.g., ANA, anti-dsDNA) parameters were also investigated, and compared with the IL-23/IL-17 axis parameters using multivariate and false discovery rate (FDR) analyses.

MATERIALS AND METHODS

Recruitment of SLE Patients and Healthy Controls

According to published flow cytometry and/or ELISA case-control studies utilizing peripheral blood samples to investigate IL-17 or IL-23 parameters in SLE patients compared with controls (31–35), sample size calculation was performed according to the difference of means between two independent groups using the software G*Power (version 3.1.9.3). A two-tailed hypothesis, α-error probability of 0.05, power (1-β error probability) of 0.80, effect size of 0.6, allocation ratio (N2/N1) of 1, and dropout rate of 10% were adopted. This yielded a total sample size of 100 participants divided equally between the healthy controls and SLE patients group (n = 50 per group). Thus, in this cross-sectional case-control study, 100 participants were recruited consisting of 50 established SLE patients who fulfilled the 2012 Systemic Lupus International Collaborating Clinics (SLICC) criteria (36) for SLE diagnosed from the year 2012 onwards, or the 1997 Update of the 1982 American College of Rheumatology Revised Criteria for Classification of SLE (37) for patients diagnosed before 2012, and 50 healthy control volunteers. Subject recruitment was conducted based on the inclusion and exclusion criteria (**Supplementary Table 1**). The selection of SLE patients and healthy controls for this study was conducted on the basis of random sampling. The clinico-demographic data of the patients were obtained from the Records Unit of Hospital Universiti Sains Malaysia (HUSM). SLEDAI-2K score for each patient was assigned by the attending clinician at the time of this study (i.e., on the same day when the samples were taken) according to the SLEDAI-2K score calculator (https://qxmd.com/calculate/calculator_335/sledai-2k) (38). Data on the current treatments received (i.e., prednisolone, immunosuppressants, or anticonvulsant) were obtained at the time of the study. Recruitment of the subjects and retrieval of their data were conducted from November 2018 to May 2019. The study procedures were approved by the Human Research Ethics Committee of Universiti Sains Malaysia (JEPeM) (approved ethics code: USM/JEPeM/17120680). All procedures carried out in this study involving peripheral blood samples were in accordance with the 1964 Declaration of Helsinki and its later updates, and with the institutional ethical standards. All participants were briefed about the study during the recruitment, and both verbal and written informed consents were obtained from each participant after detailed explanations of the study. The documented written informed consent permitted the investigators to conduct the recruitment, draw the blood of the participants for the experiments, and publish the results. All samples were labeled anonymously, and all data were recorded, stored, and analyzed anonymously to ensure that none of the private information such as the name of participants were disclosed. All experimental protocols were conducted according to the institutional relevant guidelines and regulations.

Serum and PBMCs Isolation

A total of 10 mL of peripheral blood was withdrawn from the healthy controls (n = 50) and SLE patients (n = 50). Of this, 6 mL were allocated in a clot activator tube for serum collection and 4 mL was placed in a BD Vacutainer® EDTA tube (Becton Dickinson, USA) for peripheral blood mononuclear cells (PBMCs) isolation. The collected blood samples were left in an upright position for 30 minutes at room temperature (RT) prior to centrifugation at 1,000 x g at 4°C for 15 min to remove the blood clot. The serum obtained from centrifugation was aliquoted immediately into 1.5 ml microcentrifuge tubes which were labeled with the identification number of the subject and the intended tests (e.g., ANA, anti-dsDNA, CRP, IL-17, IL-23), and stored at -20°C. On the other hand, PBMCs were isolated from peripheral blood by standard Histopaque-1077 (Sigma-Aldrich, USA) density centrifugation. The collected peripheral blood was inverted and left in an upright position for 30 min at RT. Four milliliters of Histopaque-1077 was transferred into a new falcon tube followed by 4 mL of peripheral blood from the EDTA tube layered carefully on top of the Histopaque-1077 with a 1:1 ratio. The blood mixture was then centrifuged at 400 x g (30 min at RT, brakes off). The PBMCs layer obtained was transferred carefully using a Pasteur pipette into a new falcon tube containing 2 mL of PBS. The mixture was then added with PBS to a total of 10 mL, mixed gently, and resuspended. The supernatant obtained after being centrifuged at 200 x g for 10 min was discarded and the pellet was resuspended using 5 mL of PBS and centrifuged. The washing process was then repeated with 500 µl of PBS before cell counting using a hemocytometer.

Flow Cytometry Analysis for IL-17RA$^+$ and IL-23R$^+$ Th Cells

A total of 5 mL of isolated PBMCs were centrifuged at 1,000 rpm for 10 min. The cells were washed with 1 ml of cold FBS followed by centrifuging at 1,000 rpm for 15 min. Then, cells were resuspended with cold FBS to a final concentration of 2 x 10^6 cells/ml. The cell mixtures were distributed and 50 µl of cell suspension was aliquoted into a microcentrifuge tube. Fluorochrome-labeled antibodies for CD3 (APC-Cy7; cat no. 560176), CD4 (PE-Cy7; cat no. 557852), IL-17RA (BB700/PerCP-Cy5.5; cat no. 747946), IL-23R (FITC; cat no. FAB14001F), and isotype controls (APC-Cy7; cat no. 560167, PE-Cy7; cat no. 557872, BB700/PerCP-Cy5.5; cat no. 566404, FITC; cat no. GZ-IC0041F) (Beckton Dickinson, USA) were used at 5 µl per test whereby the antibody concentration for each test was 0.0182 µg/µl in a final volume of 55 µl, corresponding to 1 µg of antibody per test according to the protocols of the manufacturer. Each antibody was added accordingly into the cells mixture and incubated on ice protected from light for 30 min. Washing steps were conducted twice with 1 ml of FBS, the tube inverted, and supernatant blotted from the cell pellets. The cell pellets were resuspended in 500 µl of FBS. The stained cells were then transferred into an FACS tube and analyzed using the FACS CANTO II (Beckton Dickinson). The results were acquired with the FACS Diva software (Beckton Dickinson) and analyzed with FlowJo (v10; TreeStar, USA).

ELISA for Serum IL-17 and IL-23 Levels

ELISA tests were conducted according to manufacturer's instructions (BioLegend, USA). Briefly, monoclonal antibodies specific for human IL-17 (cat no. 433917) and IL-23 (cat no. 437607) were pre-coated onto the provided 96-well strip microplate. Standards and samples for both IL-17 and IL-23 levels detection were run in duplicate, and the standard curve was obtained for each assay. All reagents were brought to RT prior to use, and 50 ml of 20X wash buffer was diluted in 950 ml of sterilized dH$_2$O to prepare a 1X wash buffer. Each microplate used was labeled accordingly, and the washing process to remove unbound materials was conducted with 300 µl of 1X wash buffer per well, and the residual buffer was blotted by tapping firmly the microplate upside down on a clean absorbent paper. The final washing process was conducted for 30 seconds to 1 min for each wash to minimize the background. Absorbance values were recorded at 450 nm and 570 nm by the ELISA microplate reader (Tecan, Switzerland).

Anti-Nuclear Antibodies Indirect Immunofluorescence Assay (IIFA)

The IIFA for the AESKUSLIDES ANA-HEp-2 test was performed according to the instructions of the manufacturer (AESKU DIAGNOSTICS, Germany). All components of AESKUSLIDES-ANA-Hep-2 were allowed to reach RT before usage. Serum samples were diluted following the ratio of 1:80 with PBS (20 µl of serum + 1,580 µl of PBS), and 25 µl of each diluted serum and controls (ready-to-use format) were pipetted into the appropriate wells. Slides were incubated in a humidified chamber for 30 min at RT. The slides were subsequently rinsed with the 1X wash buffer using a squeeze wash bottle followed by 10 min of washing in a glass staining dish containing fresh wash buffer. The slides were then transferred to the humidified chamber and excess wash buffer was carefully removed by filter paper. Subsequent incubation with secondary antibody conjugated with FITC was conducted in the humidified chamber for 30 min at RT in the dark. Washing steps were then repeated before a coverslip was mounted on each slide. The specific green fluorescent staining of the antigen-antibody complex was observed and interpreted with an Olympus BX-60 fluorescence microscope (Olympus, Tokyo, Japan). Interpretation of the ANA staining patterns were conducted by immunologists (KKW, CMCH) according to the International Consensus on ANA Patterns (ICAP; http://www.anapatterns.org/) nomenclature and definitions of 29 HEp-2 IIFA patterns, where each pattern is ascribed with an alphanumeric code (AC-1 to AC-29) (39).

Anti-dsDNA ELISA Test

ImmuLisa™ Anti-dsDNA Antibody Enhanced ELISA tests were conducted according to the instructions of the manufacturer (Immco Diagnostics, USA). Anti-dsDNA IgG antibodies were detected by the test. Firstly, the microwells were coated with purified dsDNA antigen followed by blocking steps to reduce non-specific binding. Controls, calibrators, and patient sera were incubated in the antigen-coated wells, allowing the specific

antibody in the serum to bind to the dsDNA antigens. Bound antibodies were detected by adding the anti-human IgG conjugate enzyme to the microwells and the presence of the antibodies were detected by a color change produced after a specific enzyme substrate (TMB) were added. After the washing process, to remove the unbound antibody and conjugate, the reaction was stopped and the intensity of the color change was detected by a spectrometer at 450 nm. Linear measurement was recorded and the results were reported as anti-dsDNA antibody positive or negative.

C-Reactive Protein Immunoturbidimetric Test

QuikRead go CRP test (Orion Diagnostica, Finland) is an immunoturbidimetric test based on microparticles coated with anti-human CRP F(ab)$_2$ fragments, and the test was carried out according to manufacturer's instructions. The CRP present in the serum reacts with the microparticles resulting in the turbidity of the solution as quantitatively measured by the QuikRead go instrument.

Statistical Analysis

The categorical and numerical data for the clinico-demographical and immunological characteristics of SLE patients and healthy controls were described using frequency (%) and median (interquartile rage; IQR), respectively. Normality of data was tested using the Shapiro-Wilk test. For comparison of numerical variables between two groups, unpaired t-test or Mann-Whitney test was used for normally and not normally distributed variables, respectively. Kruskal-Wallis test and the subsequent Dunn's multiple comparisons *post hoc* test were used to compare numerical variables in more than two groups. For correlation analysis between two sets of numerical variables, Pearson and Spearman correlation coefficient was used for normally and not normally distributed variables, respectively. For categorical variables, their associations were compared using the Chi-square test and Fisher's exact test (used when >20% of cells have expected frequencies below five) (40). Statistical analysis was performed using the GraphPad Prism (v9; GraphPad Software Inc., USA). Multivariate analysis of IL-23R$^+$ Th cells combined with age or prednisolone intake *versus* SLEDAI-2K score groups (i.e., SLEDAI-2K score 0 or ≥1) was conducted using multiple logistic regression (IBM SPSS v22; SPSS Inc., Chicago, IL, USA). For all tests, two-tailed $p<0.05$ was considered statistically significant.

RESULTS

Clinico-Demographical Characteristics of the Participants

A total of 50 SLE established patients and 50 healthy controls were recruited in this study. The SLE patients and healthy controls were mostly female (98% and 82%, respectively). Majority of the SLE

patients were ethnic Malay (n = 46/50; 92%) followed by non-Malay (n = 4/50; 8%), and the same was observed for the healthy controls with 82% (n = 41/50) Malay and 18% (n = 9/50) non-Malay. In our SLE cohort, patients aged 31–40 years were the most common, followed by 21–30 years and other age groups (**Supplementary Figure 1**). A number of SLE patients (n = 13/50; 26%) had a family history of autoimmune diseases while none in the healthy controls group. Among the SLE patients and healthy controls enrolled in this study (n = 100), two healthy controls were smokers (n = 2/50; 4%) and all SLE patients were non-smokers. The SLEDAI-2K scores of SLE patients were calculated and recorded with a median score of 1.50 (IQR: 0–6.50). In terms of medications, 90% (n = 45/50) of SLE patients took hydroxychloroquine, followed by corticosteroid (n = 37/50; 76%), other immunosuppressants (n = 21/50; 42%), and anticonvulsant (n = 1/50; 2%) (**Table 1**).

Immunological Characteristics of SLE Patients

The patients had their SLE diagnosis established previously where all patients were positive for ANA, and 36 out of 43 (83.7%; anti-dsDNA test was not initially conducted for 7 patients) were positive for anti-dsDNA antibody leading to their SLE diagnosis according to the established criteria. In the current study, ANA and anti-dsDNA tests were conducted anew along with the CRP test. The proportion of SLE patients according to their ANA (n = 45/50 ANA-positive; 90%), anti-dsDNA (n = 36/50 anti-dsDNA-positive; 72%), or CRP status is presented in **Figure 1A**. The proportion of SLE patients positive for ANA (n = 45/50; 90%) was higher than those negative for ANA (n = 5/50; 10%, i.e., ANA seroconversion to ANA-negative post-treatment). The ANA and anti-dsDNA test results of SLE patients prior to disease diagnosis and current ANA and anti-dsDNA results as well as CRP levels are summarized in **Supplementary Table 2**.

Ten distinct ANA patterns implicated in the SLE diagnosis were observed in our cohort of SLE patients where most of the cases displayed a nuclear large/coarse speckled pattern (AC-5; n = 19/45; 42.2%), followed by nuclear homogeneous (AC-1; n = 7/45; 15.6%), nuclear fine speckled (AC-4; n = 7/45; 15.6%), smooth nuclear envelope (AC-11; n = 5/45; 11.1%), homogeneous nucleolar (AC-8; n = 2/45; 4.4%), and one case each (2.2%) for nuclear dense fine speckled (AC-2), centromere (AC-3), PCNA-like (AC-13), cytoplasmic dense fine speckled (AC-19), and NuMA-like (AC-26). Representative fluorescent images for each of these 10 ANA patterns are shown in **Figure 1B**, and fluorescent images for the rest of the ANA-positive and ANA-negative cases are shown in **Supplementary Figure 2**. The detailed ANA pattern interpretations for each SLE case are described in **Supplementary Table 3**. The proportions of the different ANA patterns observed in our cohort of cases are comparable with those of published studies utilizing the ICAP nomenclature (**Supplementary Table 4**). A total of 68.9% (n = 31/45) of the ANA-positive SLE patients were also positive for the anti-dsDNA antibody. Majority of the SLE patients had normal CRP levels (n = 28; 56%) compared with patients

TABLE 1 | Clinico-demographical and immunological characteristics of SLE patients and healthy controls.

Characteristics[a]	SLE patients (n = 50)	Healthy Controls (n = 50)
Median age (years) (IQR)	34.0 (26.0–40.8)	26.5 (24.0–32.0)
Gender		
Female	49 (98.0)	41 (82.0)
Male	1 (2.0)	9 (18.0)
Ethnicity		
Malay	46 (92.0)	41 (82.0)
Non-Malay	4 (8.0)	9 (18.0)
Family history of autoimmune disease		
Yes	13 (26.0)	0 (0.0)
No	37 (74.0)	50 (100.0)
Smoking status		
Yes	0 (0.0)	2 (4.0)
No	50 (100.0)	48 (96.0)
SLEDAI-2K (median) (IQR)	1.50 (0–6.50)	NA
Use of medicines		
Corticosteroids		
Prednisolone	38 (76.0)	NA
Immunosuppressant		
Azathioprine	20 (40.0)	NA
Hydroxychloroquine	45 (90.0)	NA
Methotrexate	1 (2.0)	NA
Anticonvulsant		
Sodium valproate	1 (2.0)	NA
Anti-nuclear antibody (ANA)		
Positive	45 (90.0)	NA
Negative	5 (10.0)	NA
Anti-dsDNA antibody, (n = 50)		
Positive (≥50 IU/ml)	36 (72.0)	NA
Negative (<50 IU/ml)	14 (28.0)	NA
C-reactive protein (CRP)		
Normal (<3 mg/l)	28 (56.0)	NA
Mildly elevated (3 ≤ x < 10 mg/l)	16 (32.0)	NA
Elevated (≥10 mg/l)	6 (12.0)	NA

[a]Data are presented as number (percentage) unless stated otherwise.

presented with mildly elevated (n = 16; 32%) or elevated (n = 6; 12%) CRP levels (**Table 1**).

Determination of IL-17RA+ and IL-23R+ Th Cells Populations by Flow Cytometry

The peak of flow cytometry histogram for unstained lymphocytes were fixed at 1 for all fluorochromes (PE-Cy7, APC-Cy7, PerCP, and FITC). To observe a distinct peak between the negative and positive cells populations, the histograms of isotype control (red shaded) and fluorochrome-stained (blue shaded) were overlapped for each fluorochrome to distinguish the negative and positive cells populations (**Supplementary Figure 3**). The gating strategy for CD3+CD4+ Th cells and subsequent determination of IL-17RA+ or IL-23R+ Th cells subset is summarized in **Supplementary Figure 4**.

IL-17RA+ and IL-23R+ Th Cells Populations in SLE Patients *Versus* Healthy Controls

The proportion of CD3+CD4+ Th cells in SLE patients was significantly higher compared with the healthy controls (28.30% vs 23.35%) (p = 0.003). Moreover, the proportions of IL-17RA+ (39.45% vs 23.65%; $p = 1.12 \times 10^{-4}$) and IL-23R+

(90.95% vs 33.25%; $p = 1.98 \times 10^{-29}$) Th cells were significantly higher in SLE patients compared with the healthy controls (**Figure 2**).

By taking into account only Malay subjects (SLE n = 46 vs healthy controls n = 41), the proportions of IL-17RA+ (39.45% vs 22.90%; $p = 2 \times 10^{-4}$) and IL-23R+ (90.95% vs 33.20%; $p = 1.75 \times 10^{-25}$) Th cells remained significantly higher in SLE patients compared with the healthy controls (**Supplementary Figure 5**).

Serum IL-17 and IL-23 Levels in SLE Patients and Healthy Controls

Serum IL-17 levels were significantly lower in SLE patients compared with the healthy controls (4.63 vs 5.16 pg/ml; $p = 8.32 \times 10^{-5}$). The serum levels of IL-23 did not differ significantly between the SLE patients and healthy controls (22.85 vs 25.23 pg/ml; p = 0.728) (**Figure 3**). Higher serum IL-17 levels were significantly and positively associated with the proportion of IL-17RA+ Th cells in SLE patients (r = 0.294; p = 0.038). However, these associations were not observed in the healthy controls. In addition, serum IL-17 and IL-23 levels were not associated with the proportion of IL-23R+ Th cells in both the healthy controls and SLE patients (**Figure 4**).
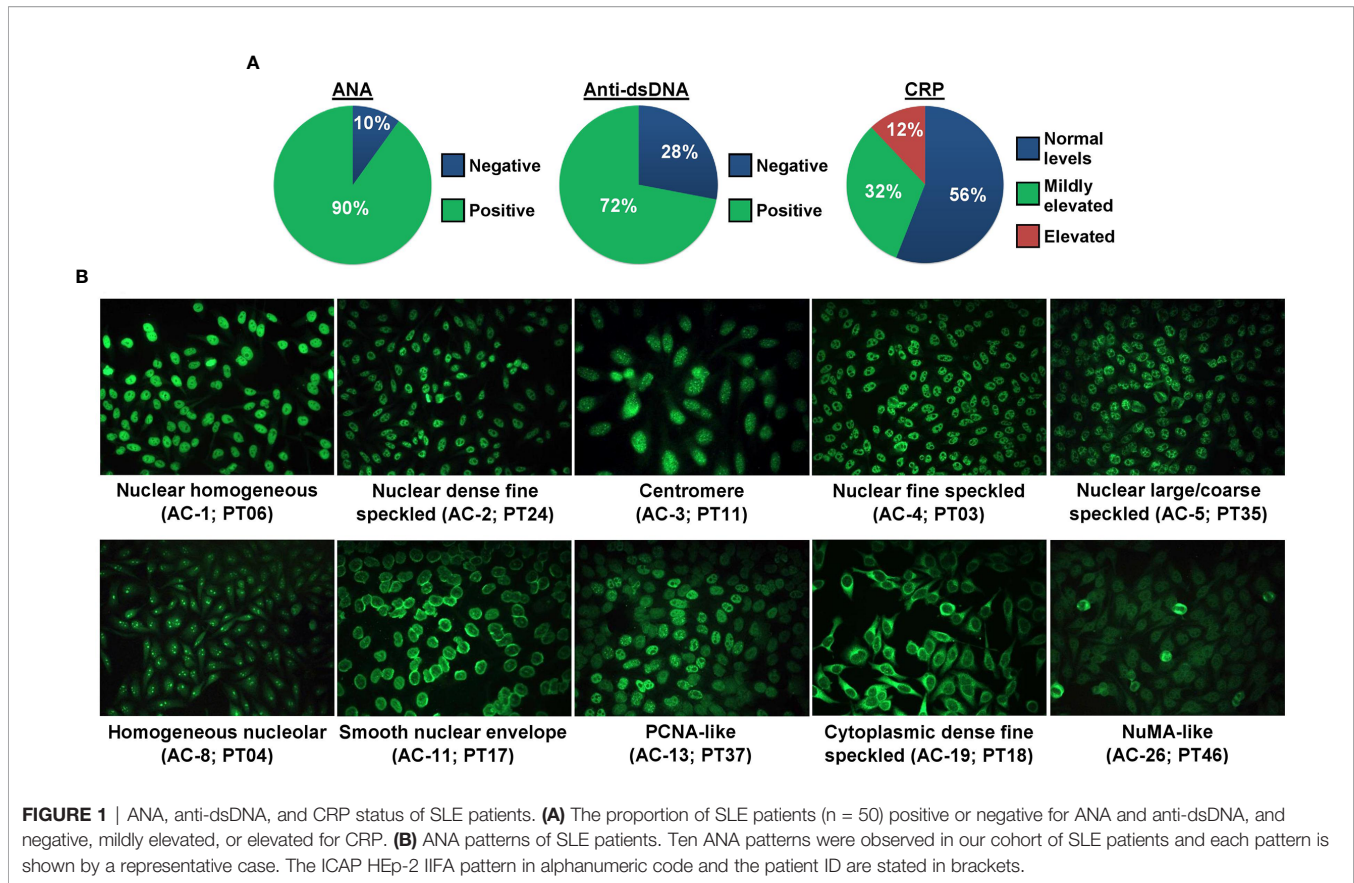
Higher Proportion of IL-23R+ Th Cells, Younger Age, and Prednisolone Intake Were Associated With Higher SLEDAI-2K Scores

Next, we assessed the association of the clinico-demographical and immunological characteristics with the SLEDAI-2K scores, i.e., SLEDAI-2K score 0 (n = 23) vs ≥1 (n = 27) in SLE patients. Younger SLE patients (36 vs 30 years old; p = 0.042), prednisolone intake (56.5% vs 88.9%; p = 0.009), and higher proportion of IL-23R+ Th cells (89.10% vs 92.40%; p = 0.017) were significantly associated with higher SLEDAI-2K scores (**Table 2**). The rest of the parameters that were examined did not exhibit such significance with the SLEDAI-2K scores, i.e., ethnicity (p = 0.614), history of autoimmune disease (p = 0.191), immunosuppressant intake (p = 0.441), ANA-positive (p = 0.651), anti-dsDNA-positive (p = 0.324), CRP status (p > 0.999), serum IL-17 levels (p = 0.508), serum IL-23 levels (p = 0.466), and the proportion of IL-17RA+ Th cells (p = 0.743) (**Table 2**).

In addition, we also examined the association between serum IL-17 and IL-23 levels, and IL-17RA+ or IL-23R+ Th cells population with the immunological characteristics in SLE patients. No significant association was observed in any of the comparisons made between serum IL-17 or IL-23 levels, and IL-17RA+ or IL-23R+ Th cells proportion with ANA, anti-dsDNA, or CRP status (**Supplementary Table 5**).

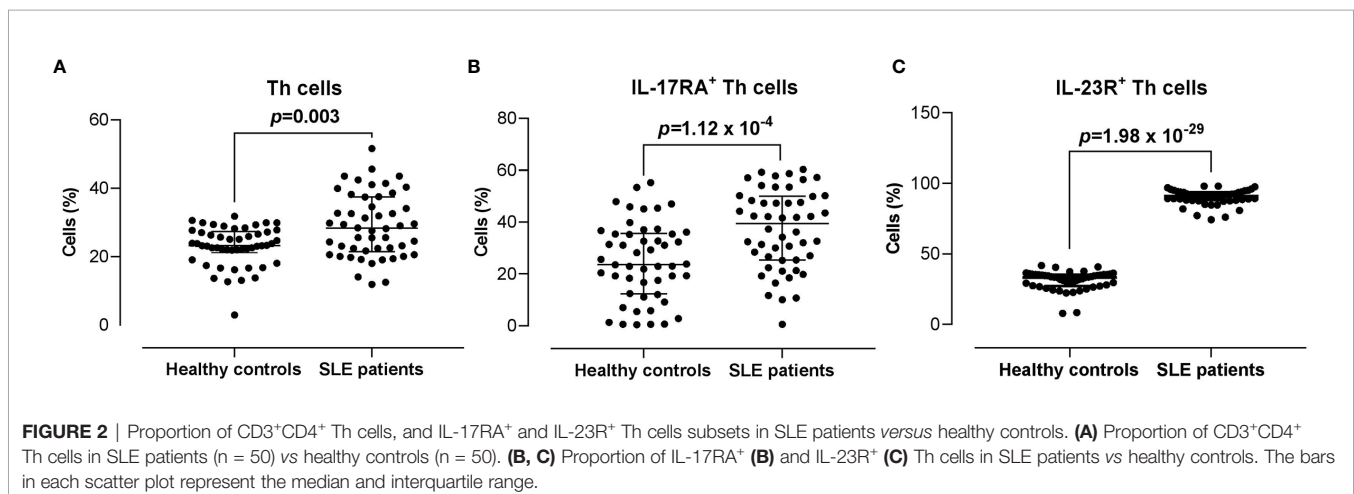## Multivariate and False Discovery Rate Analyses of the Associations With SLEDAI-2K Scores

As younger age, prednisolone intake, and the proportion of IL-23R+ Th cells were associated with higher SLEDAI-2K scores, the univariate association of IL-23R+ Th cells proportion with age or prednisolone intake was first assessed.

FIGURE 1 | ANA, anti-dsDNA, and CRP status of SLE patients. **(A)** The proportion of SLE patients (n = 50) positive or negative for ANA and anti-dsDNA, and negative, mildly elevated, or elevated for CRP. **(B)** ANA patterns of SLE patients. Ten ANA patterns were observed in our cohort of SLE patients and each pattern is shown by a representative case. The ICAP HEp-2 IIFA pattern in alphanumeric code and the patient ID are stated in brackets.

Higher IL-23R⁺ Th cells proportion was significantly associated with a younger age ($p = 0.025$) (**Figure 5A**), and no significant association was observed for prednisolone intake (**Figure 5B**). Multivariate analysis of IL-23R⁺ Th cells and age or prednisolone intake *versus* SLEDAI-2K scores demonstrated that IL-23R⁺ Th cells proportion was not significantly associated with higher SLEDAI-2K scores when age was taken into account (OR: 1.10, 95% CI: 0.97–1.25; $p = 0.146$). However, IL-23R⁺ Th cells proportion remained significantly associated with higher SLEDAI-2K scores when prednisolone intake was taken into account (OR: 1.18, 95% CI: 1.02–1.36; $p = 0.027$) (**Figure 5C**).

A total of 36 associations were tested for a potential significance in this study where 6 of the associations demonstrated significant p-values. To account for multiple hypothesis testing, the p-values of these associations were corrected using the Benjamini-Hochberg (BH) FDR procedure. The threshold to use for significance for the generated q-values (BH-corrected p-values) was 0.25 (41, 42). Two of the six
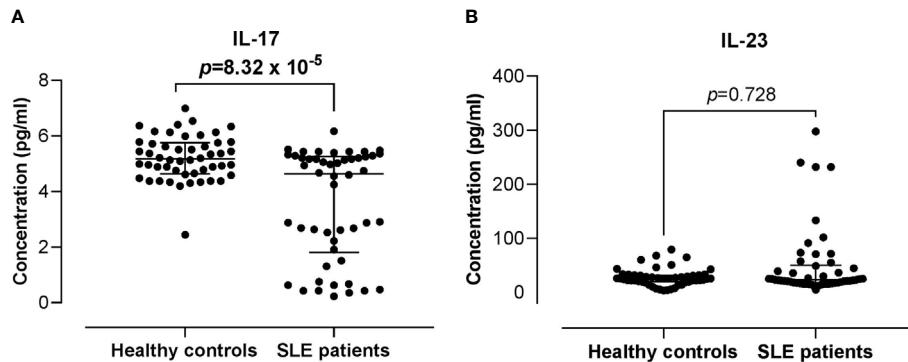

FIGURE 2 | Proportion of CD3⁺CD4⁺ Th cells, and IL-17RA⁺ and IL-23R⁺ Th cells subsets in SLE patients *versus* healthy controls. **(A)** Proportion of CD3⁺CD4⁺ Th cells in SLE patients (n = 50) *vs* healthy controls (n = 50). **(B, C)** Proportion of IL-17RA⁺ **(B)** and IL-23R⁺ **(C)** Th cells in SLE patients *vs* healthy controls. The bars in each scatter plot represent the median and interquartile range.

FIGURE 3 | Comparison of the serum IL-17 and IL-23 levels between SLE patients *versus* healthy controls. **(A)** IL-17 serum levels in SLE patients *vs* healthy controls. **(B)** IL-23 serum levels in SLE patients *vs* healthy controls. The bars in each scatter plot represent the median and interquartile range.

significant associations were rendered insignificant through BH correction, i.e., serum IL-17 *vs* IL-17RA$^+$ Th cells in SLE patients ($q = 0.252$), and age *vs* SLEDAI-2K scores ($q = 0.252$). The rest of the four associations remained significant, i.e., IL-23R$^+$ Th cells *vs* SLEDAI-2K scores ($q = 0.204$), prednisolone intake *vs* SLEDAI-2K scores ($q = 0.162$), multivariate analysis of prednisolone intake (tested with IL-23R$^+$ Th cells combination) *vs* SLEDAI-2K scores ($q = 0.162$), and multivariate analysis of IL-23R$^+$ Th cells (tested with prednisolone intake combination) *vs* SLEDAI-2K scores ($q = 0.243$) (**Supplementary Table 6**). Finally, the complete data of

the clinico-demographical and immunological characteristics, as well as the serum interleukin and their receptors data for each individual healthy control and SLE patient involved in this study, are presented in **Supplementary Table 7**.

DISCUSSION

In this study, we demonstrated that CD3$^+$CD4$^+$ Th cells were significantly higher in SLE patients compared with the healthy
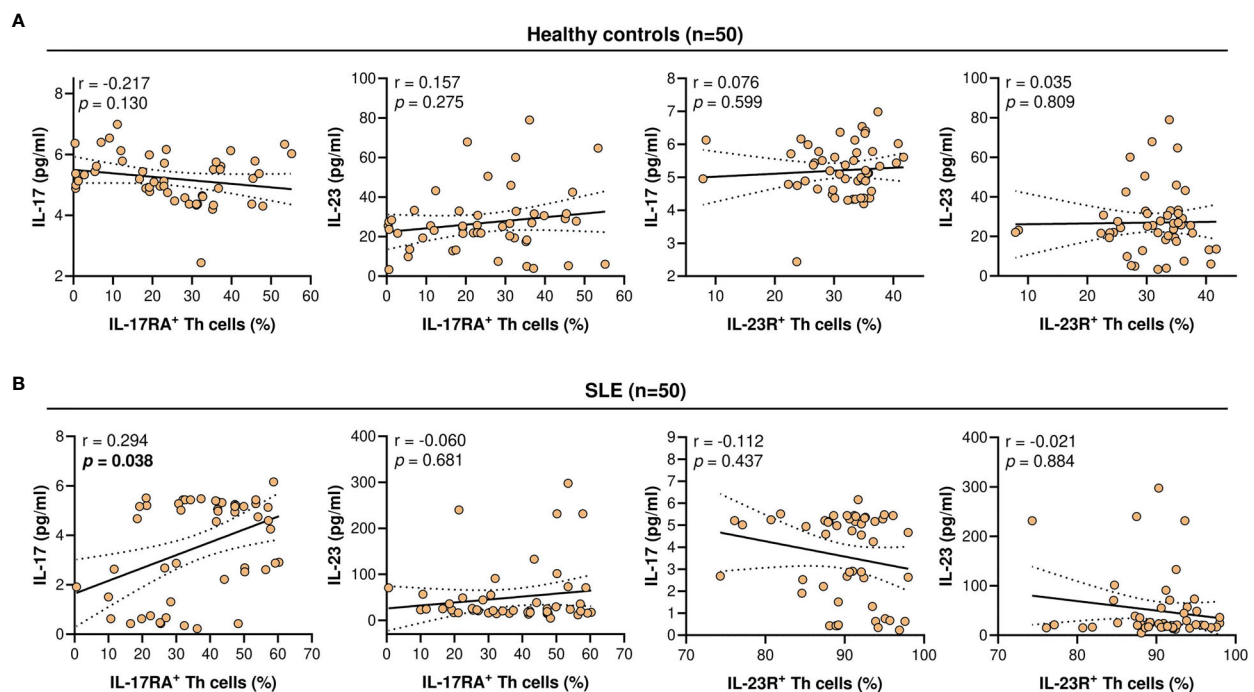

FIGURE 4 | Correlation of IL-17RA$^+$ or IL-23R$^+$ Th cells population (%) with serum IL-17 or IL-23 levels (pg/ml). **(A)** Correlation in healthy controls (n = 50). **(B)** Correlation in SLE patients (n = 50). For each graph, the linear regression plot is represented by a best-fit line surrounded by adjacent 95% confidence bands.

TABLE 2 | Association of the clinico-demographical and immunological characteristics with SLEDAI-2K scores in SLE patients (n = 50).

Characteristics		SLEDAI-2K		p-value
		0 (n = 23)	≥1 (n = 27)	
Age (years) (median; IQR)		36.0 (31.0–49.0)	30.0 (24.0–38.0)	**0.042**[a]
Ethnicity (n; %)	Malay	22 (95.7)	24 (88.9)	0.614[b]
	Chinese	1 (4.3)	3 (11.1)	
History of autoimmune disease (n; %)	Yes	8 (34.8)	5 (18.5)	0.191[c]
	No	15 (65.2)	22 (81.5)	
Prednisolone (n; %)	Yes	13 (56.5)	24 (88.9)	**0.009**[c]
	No	10 (43.5)	3 (11.1)	
Immunosuppressant (n; %)	Yes	11 (47.8)	10 (37.0)	0.441[c]
	No	12 (52.2)	17 (63.0)	
ANA (n; %)	Yes	20 (87.0)	25 (92.6)	0.651[b]
	No	3 (13.0)	2 (7.4)	
Anti-dsDNA (n; %)	Yes	15 (65.2)	21 (77.8)	0.324[c]
	No	8 (34.8)	6 (22.2)	
CRP (mg/ml) (n; %)	<3	13 (56.5)	15 (55.6)	>0.999[b]
	3≤ x <10	7 (30.5)	9 (33.3)	
	≥10	3 (13.0)	3 (11.1)	
IL-17 (pg/ml) (median; IQR)		4.56 (1.91–5.17)	4.67 (1.51–5.28)	0.508[a]
IL-23 (pg/ml) (median; IQR)		22.7 (16.43–35.55)	23.01 (18.90–57.0)	0.466[a]
IL-17RA⁺ Th cells (%) (median; IQR)		41.80 (25.40–50.20)	36.20 (25.20–47.60)	0.743[d]
IL-23R⁺ Th cells (%) (median; IQR)		89.10 (87.30–91.70)	92.40 (89.20–95.0)	**0.017**[a]

[a]Mann-Whitney test.
[b]Fisher's exact test.
[c]Chi-square test.
[d]Unpaired t-test.
p < 0.05 in bold.

controls. In terms of IL-23/IL-17 axis receptors, we observed that IL-17RA⁺ and IL-23R⁺ Th cells were significantly increased in SLE patients than in the healthy controls. This is comparable with another study where it was reported that the percentages of CD4⁺IL-23R⁺ cells from PBMCs was significantly higher in SLE patients (35). The production of IL-17 is driven by IL-23 (11, 43) and IL-23R is expressed on activated Th17 cells (44). Furthermore, the increased IL-17RA⁺ and IL-23R⁺ Th cells populations in SLE patients may be attributable to their shared molecular signaling pathway as the binding of IL-23 with its receptor complex activates STAT3 signaling in Th17 cells leading to IL-17 production through differentiation of Th17 cells (45, 46). Along with our observation of increased IL-23R⁺ Th cells in SLE patients associated with higher SLEDAI-2K scores, IL-23R⁺ Th cells may play crucial roles in the progression and aggressive disease course of SLE patients.

Multiple studies have reported significantly elevated serum IL-23 levels in SLE patients compared with healthy controls (12, 33, 47). Specifically, serum IL-23 levels were significantly higher in active SLE patients compared with inactive and healthy controls group (32, 48). However, our study demonstrated that serum IL-23 levels did not differ significantly between SLE patients and healthy controls, suggesting that increased IL-23R signaling

might be achieved *via* higher surface levels of IL-23R instead of serum IL-23 levels. On the other hand, serum IL-17 was lower in SLE patients but IL-17RA⁺ Th cells population was higher in the patients, suggesting that a higher consumption of serum IL-17 by increased IL-17RA may occur in SLE patients. This is also supported by our observation that serum IL-17 levels had a positive relationship with IL-17RA⁺ Th cells in SLE patients but not in the healthy controls. However, these hypotheses require further validation by functional and mechanistic studies such as gene knockdown or knockout methodologies.

In addition, significantly downregulated serum IL-17 levels in our cohort of SLE patients is in contrast with the findings of elevated serum IL-17 levels in SLE patients as reported previously (22–24, 26). This may also be due to the use of hydroxychloroquine, an immunosuppressant routinely used to treat SLE patients (27, 49–52), by the majority of our cohort of SLE patients (n = 45/50; 90%). It has been reported that hydroxychloroquine use resulted in a significant reduction of disease activities in SLE patients where most of the cytokines and pro-inflammatory markers were reduced following the treatment (51–54). Hydroxychloroquine has been shown to inhibit Th17 cells proliferation and differentiation along with reduced IL-17 production by Th17 cells *in vitro* (27). Essentially, SLE patients
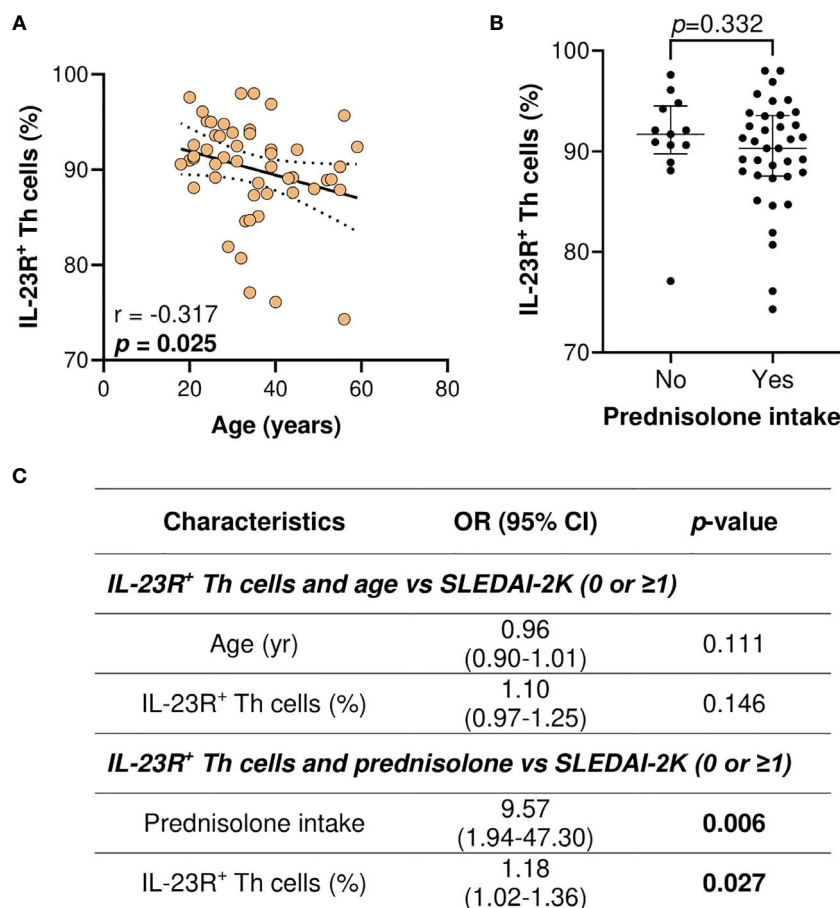
FIGURE 5 | Association of IL-23R⁺ Th cells (%), age (years), and prednisolone intake with the SLEDAI-2K scores in SLE patients. **(A)** Association of IL-23R⁺ Th cells with age in SLE patients. **(B)** Association of IL-23R⁺ Th cells with prednisolone intake in SLE patients. **(C)** Multivariate analysis of IL-23R⁺ Th cells and age or prednisolone intake *vs* SLEDAI-2K (0 or ≥1) in SLE patients (n = 50).

(n = 6) demonstrated lower levels of serum IL-17 and reduced proportion of CD3⁺CD8⁻IL-17⁺ Th17 cells after receiving 4 weeks of treatment with hydroxychloroquine and prednisone (27). Furthermore, in PBMCs derived from a cohort of 18 SLE patients, stimulation of these PBMCs with phorbol 12-myristate 13-acetate and ionomycin displayed reduced IL-17 production after treatment with hydroxychloroquine (55). Elevated serum levels of IL-17 correlated positively with the SLEDAI-2K scores in SLE patients as reported in previous studies (26, 56). In contrast, several studies demonstrated that there were no associations observed between IL-17 (21, 57) or IL-23 (58) with the SLEDAI-2K scores, similar with the findings in our study.

In our multivariate analysis, IL-23R⁺ Th cells and SLEDAI-2K association was dependent on age in our SLE cohort, tallying with the univariate comparison where younger SLE patients had a significantly higher proportion of IL-23R⁺ Th cells. These observations are comparable with independent studies in which younger SLE patients are more likely to present with nephritis and greater organ damage (59–62), and more likely to

be hospitalized with SLE as a diagnosis (59). Whether an increased proportion IL-23R⁺ Th cells plays a direct, causative role in exacerbating disease activities in younger SLE patients remains to be elucidated. On the other hand, IL-23R⁺ Th cells proportion remained significantly predictive of worse SLEDAI-2K scores when prednisolone intake was taken into account in the multivariate analysis, suggesting that prednisolone did not affect IL-23R⁺ Th cells proportion in SLE patients. This also suggests that alternative therapeutics specifically targeting IL-23R or its downstream signaling may be beneficial for patients who fail to respond to prednisolone.

In terms of immunological parameters, 10% of the established SLE patients in our cohort demonstrated ANA seroconversion. Loss of ANA over time in established SLE patients has been reported in up to approximately 30% of the patients (63–67). ANA seroconversion may reflect responses to therapies or naturally-occurring exhaustion of autoimmune clones (66), and whether our population of SLE patients displayed a reduced retention of autoimmune clones post-therapy is subject to future investigations. We observed that the routine

biomarker anti-dsDNA did not correlate with the SLEDAI-2K scores in SLE patients. This is in agreement with multiple independent reports whereby anti-dsDNA status does not show significant relationship with SLE disease activities (68–70). As anti-dsDNA antibodies do not always correlate with SLE disease activities, it is thought that anti-dsDNA encompasses a large spectrum of different chromatin targets that may explain their heterogeneity in lupus activities (70, 71). This proposal appears to tally with past observation in which IgA, but not IgG or IgM, anti-dsDNA was significantly elevated in SLE patients with higher disease activities (72). This represents fertile grounds for further investigation and validation. In terms of CRP, it has been reported that IL-17 correlates with CRP levels and other immunology diagnostic parameters including IgG and IgM (57). However, our study demonstrated that there were no associations between the interleukin parameters (i.e., IL-17, IL-23, IL-17RA⁺ Th cells, and IL-23R⁺ Th cells) with the immunological parameters investigated in this study, suggesting that the IL-17/IL-23 axis acts independently of ANA, anti-dsDNA, and CRP.

We acknowledge the limitations of the study as follows: 1) Established SLE patients were recruited whereby all patients had been on treatment, hence, a proportion of the patients demonstrated ANA seroconversion and lower disease activities; 2) A confirmatory cohort of SLE patients especially of SLE patients recruited from multiple centers is recommended to validate and expand on the findings of the current study; and 3) We did not investigate which Th cell subsets expressing IL-17RA or IL-23R were altered such as Th1 and Th17 populations. Nonetheless, we recommend this as an avenue for future investigations particularly for IL-23R.

In conclusion, our study demonstrated that increased populations of IL-17RA⁺ and IL-23R⁺ Th cells occurred in SLE patients, and IL-23R⁺ Th cells population was associated with higher SLEDAI-2K scores. Our studies support future research either in the production or clinical trials of therapeutic antibodies targeting IL-23/IL-17 axis receptors in SLE, particularly IL-23R, and the inhibition of IL-23/IL-17 receptors is a promising therapeutic strategy in this autoimmune disorder.

DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found in the article's/**Supplementary Material**.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Human Research Ethics Committee of Universiti Sains Malaysia (JEPeM) (approved ethics code: USM/JEPeM/17120680). All patients and participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

CMCH and KKW conceived the study and recruited research grants. AFI, NDMS, WSWG and CMCH recruited the samples and retrieved the clinico-demographical data of the subjects. AFI and NDMS conducted the experiments, and KKW supervised the experiments. AFI, CMCH and KKW designed the study. AFI and KKW performed data analysis, generated the figures and tables, conducted literature searches, and wrote the manuscript. KKW revised the manuscript. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2021.690908/full#supplementary-material

Supplementary Figure 1 | Distribution of age in 50 SLE patients.

Supplementary Figure 2 | ANA patterns of the rest of the SLE patients (n = 40). The ICAP HEp-2 IIFA pattern definition with alphanumeric code and patient ID are stated above and below, respectively, the fluorescent images.

Supplementary Figure 3 | The histogram of isotype control (red shaded) and single color stained (blue shaded) were overlapped to determine negative and positive populations; PE-Cy7 **(A)**, APC-Cy7 **(B)**, PerCP **(C)** and FITC **(D)**. The negative and positive populations were distinguished by the cell population count and mean for each fluorochrome where they were higher in positive population.

Supplementary Figure 4 | Gating strategy for analysis of CD3⁺CD4⁺ Th cells and IL-17RA⁺ or IL-23R⁺ Th cells subset. Lymphocytes from PBMCs were gated according to forward-scatter (FSC) and side-scatter (SSC) **(A)**, and further gated for CD3⁺CD4⁺ Th cells population, as well as IL-17RA⁺ or IL-23R⁺ Th cells subset **(B)**. Numbers in each histogram represent positive populations; IL-17RA and IL-23R were labeled with PerCP and FITC, respectively.

Supplementary Figure 5 | Proportion of IL-17RA⁺ **(A)** and IL-23R⁺ **(B)** Th cells subsets in SLE patients and healthy controls within Malay subjects.

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