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ORIGINAL ARTICLE

Modulatory Effect of the Euro-Lupus Low-Dose Intravenous Cyclophosphamide Regimen on Circulating Immune Cells in Systemic Lupus Erythematosus

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Abstract

A Euro-Lupus regimen of low-dose intravenous cyclophosphamide (CFA) is commonly used to treat severe organ manifestations of systemic lupus erythematosus (SLE), particularly lupus nephritis (LN). There are no data on the distributions and dynamics of immune cell populations in patients with various treatment outcomes. The circulating immune cells of 11 female SLE patients were assessed before and after Euro-Lupus regimen (cumulative dose of 3000 mg CFA) by flow cytometry together with those of 16 healthy women. A subanalysis was performed in LN patients who achieved complete remission (CR; $n=3$), partial remission (PR; $n=4$), and no response (NR; $n=2$). In SLE, the Euro-Lupus regimen decreased the percentage and absolute count of B cells; increased the percentage of $CD8^+$ T cells, T regulatory cells, neutrophils, and monocyte subsets; and activated T and NK cells compared to healthy controls ($P<0.050$). Patients with LN achieving CR had significantly lower proportions of $CD27^+$ B memory cells compared to poor responders (PR/NR, $P=0.035$). The post-treatment percentages and absolute numbers of B cells, T cells, NK cells, monocytes, and neutrophils showed high inter-individual variability with no association with treatment outcome. Our pilot study revealed the dynamics of changes in immune cell populations in SLE patients during a Euro-Lupus regimen, mainly the lowering of B cells. In LN patients who achieved CR, a lower proportion of $CD27^+$ B memory cells was evident compared to poor responders (PR/NR). Further studies on usefulness of monitoring immune cells for treatment response prediction on larger cohorts are needed.

Keywords SLE · Cyclophosphamide · Immunophenotyping · Flow cytometry · Treatment outcome · Lupus nephritis

Introduction

Systemic lupus erythematosus (SLE) is a chronic inflammatory autoimmune disease that mainly affects women and can impact any organ or organ system of the body (Liu and Davidson 2012). Treatment choices, which depend on the severity of the disease, the organ manifestations, and the symptoms, should control the disease's activity, prevent organ damage, and minimise the risk of side effects of prescribed drugs (Horák et al. 2013).

Despite some new therapeutic options, treatment regimens involving cyclophosphamide (CFA) remain a standard of care in guidelines for the treatment of major organ manifestations of SLE and exhibit excellent efficacy, especially in renal and central nervous system (CNS) lupus (Austin et al. 2009; Dörner et al. 2009; Fanouriakis et al. 2016). In addition to being one of the most effective immunosuppressive therapies, CFA also has immunomodulatory, antimitotic, and anti-replicative properties (Ahlmann and Hempel 2016). Nowadays, there is a growing body of evidence about the clinical benefits of the Euro-Lupus regimen of low-dose intravenous CFA (called The Euro-Lupus regimen, Euro-Lupus Nephritis Trial) over high-dose CFA (Houssiau et al.

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2002), predominantly due to associated reduced gonadal toxicity and preservation of the ovarian reserve (Tamirou et al. 2017). Although a number of studies have reported favourable clinical impacts of the Euro-Lupus regimen for SLE patients (Houssiau et al. 2002, 2010; Tamirou et al. 2017), only limited data exist on the effects on circulating immune cells. A study by Fassbinder et al. (2015) revealed that a Euro-Lupus low-dose intravenous CFA regimen increased the number of circulating $CD8^+$ effector T cells and plasmacytoid dendritic cells 3 months after treatment initiation compared to mycophenolate mofetil treatment in lupus nephritis (LN) patients. However, from studies with other CFA regimens there is evidence that other immune cells may also be influenced by this emerging treatment. A regimen using a combination of low-dose CFA with prednisolone reported a decrease of lymphocyte subsets and an increase in $CD4^+$ T cells in SLE (Zhao et al. 2012). Another study evaluated the effects of high-dose CFA therapy in patients who did not respond to previous prednisolone therapy and reported activation of T cells (Amano et al. 2000). Besides the differences in CFA doses within particular regimens, combinations with different drugs, evaluations at different time points, and investigations of different immune cell types and patient cohorts, these studies did not comprehensively describe the post-treatment changes in immune cells and did not compare patients with various treatment outcomes.

Therefore, we examined the modulating effects of a Euro-Lupus low-dose intravenous CFA regimen on the major populations of circulating immune cells and their activation status in women with organ-threatening SLE. Moreover, this study investigated for the first time the differential immune cell profiles in LN patients who, after the Euro-Lupus regimen, achieved complete remission (CR), partial remission (PR), or no remission (NR). The usefulness of differential immune cell profiles for the prediction of the treatment outcome was evaluated.

Materials and Methods

Study Population and Materials

The study cohort consisted of 11 women (median age: 42 years; min–max: 20–64 years) with major organ manifestations of SLE who were indicated for Euro-Lupus low-dose intravenous CFA regimen in the tertiary rheumatology centre (University Hospital Olomouc) in Central Moravia. All patients fulfilled both the American College of Rheumatology and the Systemic Lupus International Clinics criteria for the classification of SLE (Tan et al. 1982). Regarding the organ manifestations, nine patients had LN type III or IV, one had CNS lupus (aseptic meningitis), and one had lupus myocarditis with cardiac failure (Weening et al. 2004). Disease activity was evaluated using the Systemic Lupus Erythematosus Disease Activity Index (SLEDAI-2 K) scoring system (Gladman et al. 2000). All patients received a Euro-Lupus low-dose intravenous CFA regimen (Houssiau et al. 2002; Horák et al. 2013) consisting of six fortnightly pulses at a fixed dose of 500 mg CFA, glucocorticosteroids (0.5 mg/kg/day) and hydroxychloroquine (200–400 mg *per os*). None of the enrolled patients had long-term antibiotic therapy during the Euro-Lupus regimen treatment.

Patients with LN were further subdivided according to their treatment response: i) patients who achieved complete remission ($n = 3$, proteinuria ≤ 0.5 g/day, serum creatinine ≤ 1.4 mg/day, SLEDAI 0 or 4 in the case of serological positivity), ii) patients who achieved partial remission ($n = 4$, proteinuria ≤ 1.5 g/day, $\leq 25\%$ increase in baseline creatinine), or iii) non-responders ($n = 2$, proteinuria > 3.5 g/day, and failure of renal function) (Chen et al. 2008; van Vollenhoven et al. 2017). For the patients without LN, both achieved complete remission (symptomatic or radiologic remission in aseptic meningitis, functional and morphological recovery in myocarditis). The complete characteristics of the enrolled patients are presented in Table 1.

Peripheral blood from SLE patients was drawn 1 day prior to the initiation of the Euro-Lupus regimen, and after three time points (2 weeks after 1000, 2000, and 3000 mg cumulative doses of CFA), respectively. In each sample, complete blood counts and immunophenotyping of immune cells by flow cytometry were performed. This study also included 16 age-matched healthy female controls (median age: 41 years, min–max: 21–63 years). The control group consisted of female members of the medical staff, who provided statements attesting to their states of health and who had no autoimmune diseases in their family histories.

Written informed consent was obtained from all patients and controls, who were enrolled in accordance with the Helsinki Declaration. The study was approved by the ethics committee of the University Hospital Olomouc and Palacký University Olomouc.

Sample Processing and Immunophenotyping of Cells

Peripheral blood samples, with K3EDTA anticoagulant, were processed within 2 h after collection. The following monoclonal antibodies, conjugated with FITC, PE, PerCP-Cy5.5, PE-Cy7, APC and APC-Cy7 (all BioLegend, San Diego, CA, USA), were used for flow cytometry immunophenotyping: anti-CD3 (clone OKT3), anti-CD4 (SK3), anti-CD8 (SK1), anti-CD11b (ICRF44), anti-CD14 (M5E2), anti-CD15 (W6D3), anti-CD16 (B731), anti-CD19 (SJ25C1), anti-CD27 (M-7271), anti-CD38 (HB-7), anti-CD54 (HA58), anti-CD62L (DREG-56), anti-CD64 (10.1), anti-CD69 (FN50), anti-CD127 (A019D5), anti-CD138

Table 1 Clinical characteristics of enrolled female SLE patients treated with the Euro-Lupus regimen

	Patients achieving CR					Patients achieving PR				Patients with NR	
	P1	P2	P3	P4	P5	P6	P7	P8	P9	P10	P11
Age at diagnosis (years)	21	25	43	64	31	20	31	58	54	50	64
Disease duration (months)	6	4	4	6	108	6	6	12	5	120	96
Organ involvement											
SLICC score at diagnosis	0	0	0	3	1	0	2	0	0	1	1
Lupus nephritis type III or IV (Y/N)	Y	Y	Y	N	N	Y	Y	Y	Y	Y	Y
CNS lupus (Y/N)	N	N	N	N	Y	N	N	N	N	N	N
Lupus myocarditis with cardiac failure (Y/N)	N	N	N	Y	N	N	N	N	N	N	N
Lupus arthritis (Y/N)	Y	Y	N	N	Y	Y	N	N	N	Y	Y
Haematologic manifestations											
Thrombocytopenia (Y/N)	N	Y	N	N	N	Y	Y	Y	N	N	N
Haemolytic anaemia (Y/N)	N	Y	Y	Y	N	N	Y	Y	N	N	N
SLEDAI score pre-treatment	22	36	27	18	20	21	18	23	10	16	16
SLEDAI score post-treatment	2	0	0	0	4	14	12	12	6	18	10
Δ SLEDAI score[a]	20	36	27	18	16	7	6	11	4	− 2	6
Cumulative dose of steroids (g)[c]	4.25	5.85	4.55	5.10	3.20	4.55	3.95	5.10	4.45	4.45	5.90
Treatment outcome (1/2/3/4)[b]	1	1	1	1	2	3	3	3	3	4	4
Absolute blood counts before and after the Euro-Lupus regimen treatment											
Total lymphocytes ($\times 10^9$ cells/L)											
Pre-treatment	1.27	1.74	1.78	0.40[d]	1.46	1.52	1.63	0.75[d]	0.39[d]	1.80	1.05
Post-treatment	0.48[d]	0.99	1.21	1.67	0.89	0.79[d]	1.30	0.99	0.42[d]	0.78[d]	0.54[d]
B cells total ($\times 10^9$ cells/L)											
Pre-treatment	0.34	0.53[d]	0.34	0.02	0.21	0.47	0.18	0.14	0.08	0.02	0.26
Post-treatment	0.06	0.06	0.12	0.05	0.02	0.09	0.06	0.06	0.01[d]	0.01[d]	0.02
$CD27^+$ B memory cells ($\times 10^9$ cells/L)											
Pre-treatment	0.04	0.04	0.04	0.00	0.04	0.13	0.02	0.04	0.02	0.01	0.02
Post-treatment	0.01	0.01	0.02	0.03	0.01	0.04	0.02	0.03	0.00[d]	0.00[d]	0.01
$CD27^-$ B naive cells ($\times 10^9$ cells/L)											
Pre-treatment	0.30	0.48[d]	0.30	0.01	0.16	0.33	0.16	0.09	0.06	0.01	0.24
Post-treatment	0.05	0.05	0.10	0.02	0.02	0.05	0.04	0.02	0.01	0.01	0.01
T cells total ($\times 10^9$ cells/L)											
Pre-treatment	0.86	0.88	1.17	0.33[d]	0.81	0.84	1.24	0.40[d]	0.25[d]	1.29	0.68
Post-treatment	0.33[d]	0.77	1.01	1.39	0.51	0.50	0.92	0.78	0.25	0.67	0.44[d]
Monocytes ($\times 10^9$ cells/L)											
Pre-treatment	0.67	1.25[d]	1.02	0.63	0.86	1.11	0.33	0.24	0.46	0.52	0.50
Post-treatment	2.85[d]	2.88[d]	0.50	1.02	0.59	0.81	0.43	0.49	0.41	0.28	0.92
Neutrophils ($\times 10^9$ cells/L)											
Pre-treatment	5.93	7.74[d]	4.28	11.0[d]	7.72[d]	12.9[d]	2.07	2.56	7.99[d]	2.72	2.29
Post-treatment	8.96[d]	5.88	3.52	7.10[d]	8.67[d]	14.4[d]	6.83	4.19	6.12	2.39	8.43[d]

Y yes, *N* no, *CR* complete remission, *PR* partial remission, *NR* no response, *SLICC score* Systemic Lupus International Collaborating Clinics score

[a]Δ SLEDAI score calculated as the difference between pre- and post-treatment values

[b]Treatment outcome defined by SLEDAI-2 K: (1) complete clinical and serological remission; (2) clinical remission, serological activity; (3) partial remission; (4) no remission/non-responders. Complete clinical remission was defined as follows: proteinuria < 0.5 g/day, symptomatic or radiologic remission in aseptic meningitis, functional and morphological recovery in myocarditis

[c]The cumulative dose of steroid within the Euro-Lupus regimen did not differ between patients with various treatment outcomes ($P > 0.05$)

[d]Values outside the physiological ranges

(MI15), anti-HLA-DR (L243), anti-CD3/CD16 + CD56 cocktail (UCHT1/3G8 + MEM-188). Isotype-matched antibodies (all clone MOPC-21, BioLegend, San Diego, CA, USA), conjugated with FITC, PE, PerCP-Cy5.5, Pe-Cy7, APC and APC-Cy-7, were used as negative staining controls. The panels used for immunophenotyping of particular populations are given in Table 2.

The immunophenotyping was performed as reported previously (Manukyan et al. 2017). Briefly, 50 µL of whole blood was incubated with monoclonal antibodies for 20 min in the dark at room temperature. The red blood cells were lysed with 2 mL of FACS lysing solution (diluted 1:10 with distilled water; Becton–Dickinson, San Jose, CA, USA) and washed with phosphate-buffered saline containing 1% bovine serum albumin. After identifying the cells, using their forward and side scatter characteristics (FSC, SSC, respectively), a minimum of 10,000 events was recorded using six-color flow cytometry (BD FACSCanto II, BD FACSDiva software, Becton–Dickinson, San Jose, CA, USA). The acquired data were analysed using FlowJo vX0.7 software (FlowJo LLC, Ashand, OR, USA).

The results are expressed as a percentage and mean fluorescence intensity (MFI) of cells for each marker under investigation. The absolute counts of the cell subpopulations were calculated by multiplying the percentage (results of flow cytometry) by the absolute cell counts (results of complete blood counts).

Table 2 Immunophenotyping panels used for the identification of the immune cell subpopulations and their activation in peripheral blood

Cell population	Markers
B cells	$CD3^-/CD19^+$
$CD27^+$ B memory cells	$CD3^-/CD19^+/CD27^+$
$CD27^-$ B naive cells	$CD3^-/CD19^+/CD27^-$
Plasma cells	$CD3^-/CD19^+/CD38^+/CD138^+$
$CD4^+$ T cells	$CD3^+/CD4^+/HLA\text{-}DR^+$
$CD8^+$ T cells	$CD3^+/CD8^+/HLA\text{-}DR^+$
T regulatory cells[a]	$CD4^+/CD25^{high}/CD127^-$
NK cells	$CD3^-/CD16^+/CD56^+/CD69^+$
Monocytes	$CD11b^+/CD14^+/CD64^+/HLA\text{-}DR^+$
Classical monocytes	$CD14^+/CD16^-/HLA\text{-}DR^+$
Intermediate monocytes	$CD14^+/CD16^+/HLA\text{-}DR^+$
Non-classical monocytes	$CD14^{dim}/CD16^+/HLA\text{-}DR^+$
Neutrophils	$CD11b^+/CD15^+/CD16^+/CD54^+/CD62L^+/CD64^+$

[a]Percentage of T regulatory cells was calculated from $CD4^+$ T cells; in selected samples, the intracellular staining using monoclonal antibody against FoxP3 marker was performed to identify the T regulatory population, which was overlapped with the population gained using listed surface markers

Statistical Analyses

Statistical analyses (Mann–Whitney *U* test and paired *t* test) were performed using the R statistical software package (http://www.r-project.org/). *P* values < 0.05 were considered significant.

Results

The Differences in Immune Cell Profiles Between Healthy Controls and Treated SLE Patients

To assess the differences in immune cell profiles between the healthy controls and the treated SLE patients, we compared the distribution of major cell populations in the peripheral blood between healthy controls and SLE patients before and after the Euro-Lupus regimen (a cumulative dose of 3000 mg CFA).

When comparing the immune cell profiles of healthy controls to SLE patients before the initiation of the Euro-Lupus regimen, increased percentage of B cells ($P = 0.004$), plasma cells ($P = 0.048$), T regulatory cells ($P = 0.001$), and neutrophils ($P = 0.002$) as well as decreased numbers of T cells ($P = 0.017$) and NK cells ($P = 0.019$) were detected in SLE patients (Table 3).

When immune cell populations in healthy controls were compared with SLE patients after the Euro-Lupus regimen, a trend of lower B cells was detected in SLE patients ($P = 0.054$), as were a decreased percentage of $CD4^+$ T cells ($P = 0.019$) and increased percentages of $CD8^+$ T cells ($P = 0.008$) and T regulatory cells ($P < 0.001$) (Fig. 1). The treatment resulted in higher percentages of HLA-DR in both $CD4^+$ and $CD8^+$ T cells ($P < 0.001$; $P = 0.005$), respectively. Also, NK cells in SLE were more activated as assessed by the expression and percentage of the CD69 marker ($P < 0.001$; $P = 0.001$) compared to controls. Regarding monocytes, treated SLE patients had increased percentages of non-classical $CD14^-CD16^+$ monocytes ($P < 0.001$) with lower percentages of HLA-DR in the given subpopulation ($P = 0.003$) (Table 3). Also neutrophils ($P < 0.001$) remained elevated in SLE patients (Fig. 1). Percentages of CD11b, CD54, and CD62 in neutrophils decreased in treated SLE patients compared with healthy controls ($P = 0.013$; $P < 0.001$; $P < 0.001$, respectively) (Table 3).

Changes in Immune Cell Profiles in SLE Patients with Various Treatment Outcomes

In the group of patients with LN, we analysed the distribution and dynamics of the circulating immune cells during the Euro-Lupus regimen and in subsets of patients according to the treatment outcome.

Table 3 Percentage and expression (MFI) of studied markers on B cells, T cells, NK cells, monocytes, and neutrophils in healthy controls (C) and SLE patients before and after the Euro-Lupus regimen treatment

Marker	C mean (95% CI)	SLE pre-treatment mean (95% CI)	SLE post-treatment mean (95% CI)	*P* value C vs SLE pre-treatment	*P* value C vs SLE post-treatment	*P* value SLE pre- vs post-treatment
B cells						
B cells total (%)	7.79 (6.81–8.80)	18.2 (11.6–24.8)	5.81 (3.61–8.01)	**0.004**	0.054	**<0.001**
$CD27^+$ B memory cells (%)[a]	23.5 (18.8–28.3)	16.9 (10.6–23.2)	33.2 (23.5–44.8)	0.073	0.121	**0.007**
$CD27^-$ B naive cells (%)[a]	76.3 (71.6–81.0)	82.8 (76.6–89.2)	66.4 (54.5–78.1)	0.073	0.120	**0.007**
Plasma cells	0.09 (0.04–0.14)	0.31 (0.14–0.49)	0.46 (0.14–0.77)	**0.048**	0.114	0.128
T cells						
T cells total (%)	72.3 (70.0–74.6)	64.5 (57.6–71.4)	73.4 (67.3–79.5)	**0.017**	0.577	**0.028**
$CD4^+$ T cells (%)	44.2 (40.3–48.2)	36.9 (30.1–43.7)	33.2 (26.2–40.2)	0.064	**0.019**	0.866
HLA-DR $CD4^+$ T cells (%)	4.77 (2.38–7.16)	9.48 (6.39–12.6)	13.9 (9.77–18.1)	**0.006**	**<0.001**	**0.016**
HLA-DR $CD4^+$ T cells (MFI)	65.2 (52.7–77.7)	77.5 (59.2–95.8)	58.6 (49.9–67.3)	0.381	0.382	0.100
$CD8^+$ T cells (%)	27.4 (22.8–32)	25.9 (21.8–30.1)	39.4 (32.4–46.3)	0.805	**0.008**	**<0.001**
HLA-DR $CD8^+$ T cells (%)	13.4 (6.68–19.8)	19.4 (11.6–27.2)	28.1 (21.3–35.0)	0.166	**0.005**	**0.004**
HLA-DR $CD8^+$ T cells (MFI)	44.8 (38.6–51.1)	81.6 (50.1–113)	64.3 (50.6–78.0)	**0.002**	**0.013**	0.891
T regulatory cells (%)	6.68 (5.76–7.61)	11.6 (8.17–15.0)	12.7 (9.64–15.8)	**0.001**	**<0.001**	0.443
NK cells						
NK cells total (%)	9.61 (7.55–11.7)	6.98 (3.39–10.6)	12.2 (6.72–17.6)	**0.019**	0.544	0.231
CD69 (%)	48.1 (29.3–66.9)	66.6 (46.7–86.5)	86.7 (78.3–95.2)	0.131	**0.001**	0.118
CD69 (MFI)	26.9 (22.5–31.4)	67.7 (37.4–98.0)	70.1 (49.6–90.5)	**0.009**	**<0.001**	0.624
Monocytes						
Monocytes total (%)	6.20 (4.91–7.49)	6.53 (4.89–8.17)	5.26 (3.98–6.54)	0.918	0.430	0.343
CD11b (%)	98.5 (95.9–101)	92.2 (86.9–97.4)	95.3 (93.1–97.4)	**0.010**	**0.005**	0.163
CD11b (MFI)	629 (396–862)	597 (451–743)	688 (591–785)	0.560	0.866	0.593
CD64 (%)	94.1 (88.3–99.9)	91.0 (86.1–96.0)	91.6 (87.8–95.3)	0.229	0.114	0.105
CD64 (MFI)	245 (194–297)	161 (129–192)	208 (166–249)	**0.008**	0.208	**0.034**
Classical monocytes (%)	78.0 (73.7–82.4)	65.0 (47.9–82.0)	74.4 (66.0–82.7)	0.160	0.782	0.336
HLA-DR classical (%)	94.4 (86.9–102)	93.5 (87.5–99.5)	89.4 (77.3–102)	0.431	0.877	0.727
HLA-DR classical (MFI)	207 (147–267)	131 (83.1–178)	180 (120–239)	0.093	0.521	**0.007**
Intermediate monocytes (%)	4.79 (2.87–6.70)	21.1 (5.59–36.7)	10.5 (3.28–17.8)	**0.002**	**0.029**	0.238
HLA-DR intermediate (%)	99.6 (98.9–100)	99.5 (99.2–99.9)	98.1 (95.5–101)	0.151	0.114	0.160
HLA-DR intermediate (MFI)	1539 (877–2201)	328 (207–449)	1043 (642–1444)	**0.001**	0.167	**0.027**
Non-classical monocytes (%)	0.48 (0.32–0.65)	2.02 (1.24–2.81)	3.28 (2.41–4.15)	**0.004**	**<0.001**	**0.020**
HLA-DR non-classical (%)	95.5 (90.2–101)	56.5 (32.8–80.3)	76.3 (64.8–87.8)	**0.004**	**0.003**	0.077
HLA-DR non-classical (MFI)	554 (375–733)	392 (71.8–712)	751 (470–1031)	0.203	0.270	0.211
Neutrophils						
Neutrophils total (%)	51.7 (49.7–63.7)	75.4 (65.3–85.6)	76.9 (71.4–82.5)	**<0.001**	**<0.001**	0.821
CD11b (%)	99.7 (99.1–100)	99.0 (98.5–99.5)	99.1 (98.6–99.5)	0.055	**0.013**	0.342
CD11b (MFI)	182 (88.6–274)	126 (91.3–161)	190 (132–248)	0.137	0.741	0.296
CD54 (%)	33.7 (16.9–50.4)	8.11 (1.00–15.2)	5.30 (0.03–10.6)	**0.008**	**<0.001**	0.243
CD54 (MFI)	16.8 (15.2–18.4)	22.0 (19.9–24.0)	23.6 (21.6–25.5)	**0.004**	**<0.001**	0.162
CD62L (%)	99.7 (99.6–99.8)	97.3 (95.1–99.4)	98.7 (98.0–99.3)	**0.001**	**<0.001**	0.273
CD62L (MFI)	711 (544–879)	348 (207–488)	537 (369–705)	**0.004**	0.142	0.226
CD64 (%)	n/a	51.8 (34.6–69.1)	62.4 (47.9–76.9)	n/a	n/a	0.314
CD64 (MFI)	n/a	43.2 (34.3–52.2)	50.2 (40.6–59.7)	n/a	n/a	0.240

n/a not available, *MFI* mean fluorescence intensity

[a]Data are missing for one SLE patient. Statistically significant results ($P < 0.050$) are shown in bold

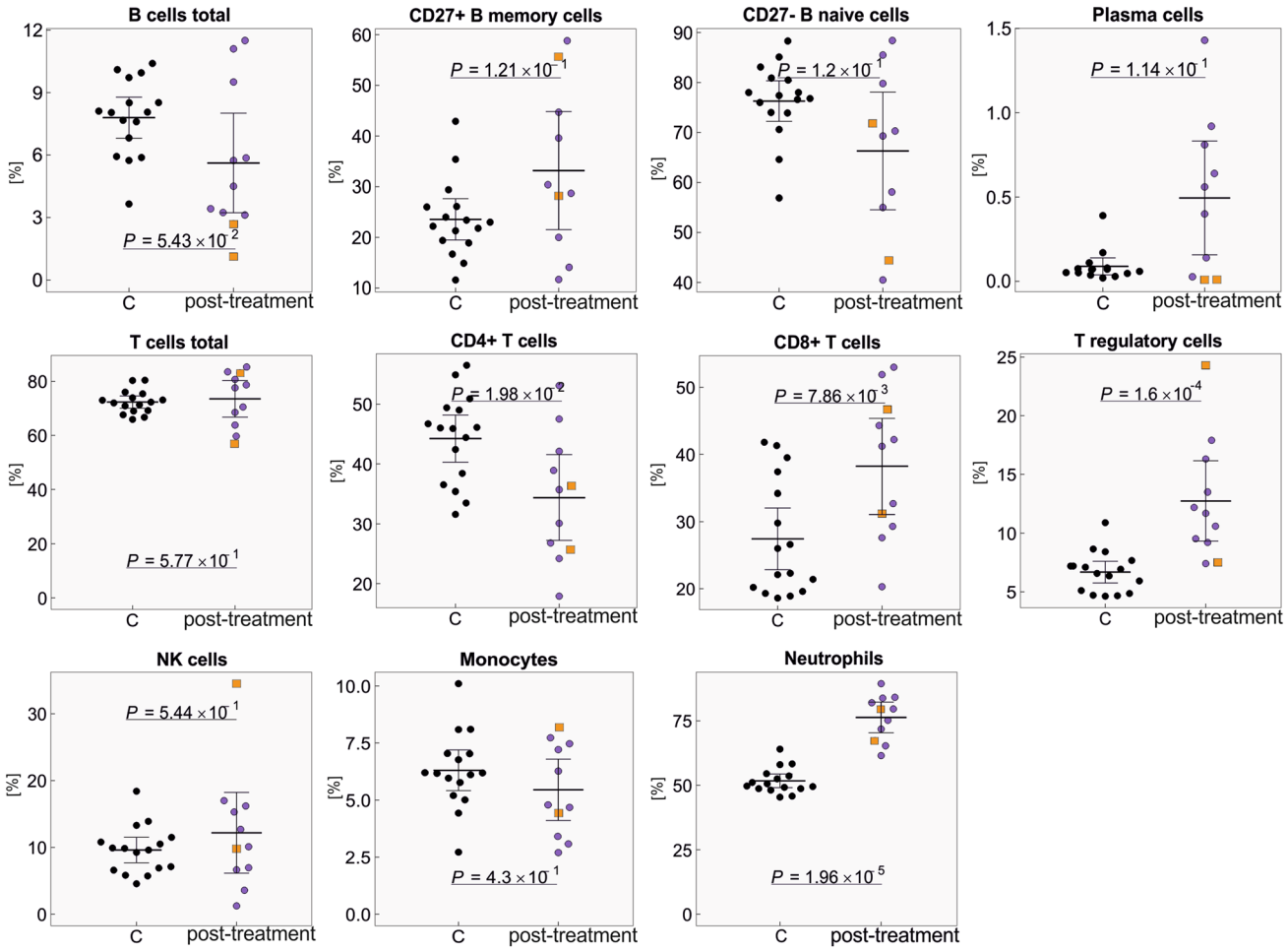

Fig. 1 Distribution of percentages in circulating immune cells in healthy controls (C) and SLE patients after the Euro-Lupus regimen treatment. In one SLE patient, $CD27^+$ B memory and $CD27^-$ B naive cells were not evaluated due to the low abundance of B cells. Circular dots represent the individual patients with LN, the square points represent the patients with CNS lupus and lupus myocarditis

The percentage of total B cells decreased during treatment with the Euro-Lupus regimen ($P=0.032$), irrespective of the treatment outcome (Figs. 2 and 3). In patients who achieved complete remission, $CD27^+$ B memory cells did not exceed 20%, which is similar to the healthy controls. In poor responders (PR/NR), the treatment resulted in an increase of $CD27^+$ B memory cells and a decrease of $CD27^-$ B naive cells (Figs. 2 and 3). The percentage of plasma cells did not change during the treatment ($P=0.213$), with no difference between good and poor responders. Regarding circulating T cells, their percentage increased during the treatment ($P=0.028$) but did not reach the difference between good and poor responders (Figs. 2 and 3). Of the T cell subsets, $CD8^+$ T cells increased during the treatment ($P=0.028$), irrespective of treatment outcome. No differences in $CD4^+$ T cells or NK cells were observed in patients with diverse treatment outcomes, although in two of the three patients who reached CR, an above-average proportion of T regulatory cells was detected (Fig. 2). The post-treatment percentages and absolute numbers of monocytes and neutrophils showed high inter-individual variability, without any association with treatment outcome.

Discussion

This study characterises changes in the immune cell subpopulations of SLE patients before and after the Euro-Lupus low-dose intravenous CFA regimen. Moreover, this study investigated for the first time the differences associated with good and poor treatment outcomes in a subgroup of SLE patients with LN. Our pilot data support the hypothesis that monitoring circulating immune cell subpopulations may be useful for predicting clinical responses.

Despite the extensive use of CFA regimens for the treatment of SLE, knowledge about their modulatory effects on circulating immune cells is limited. The majority of studies report changes in immune populations in SLE patients

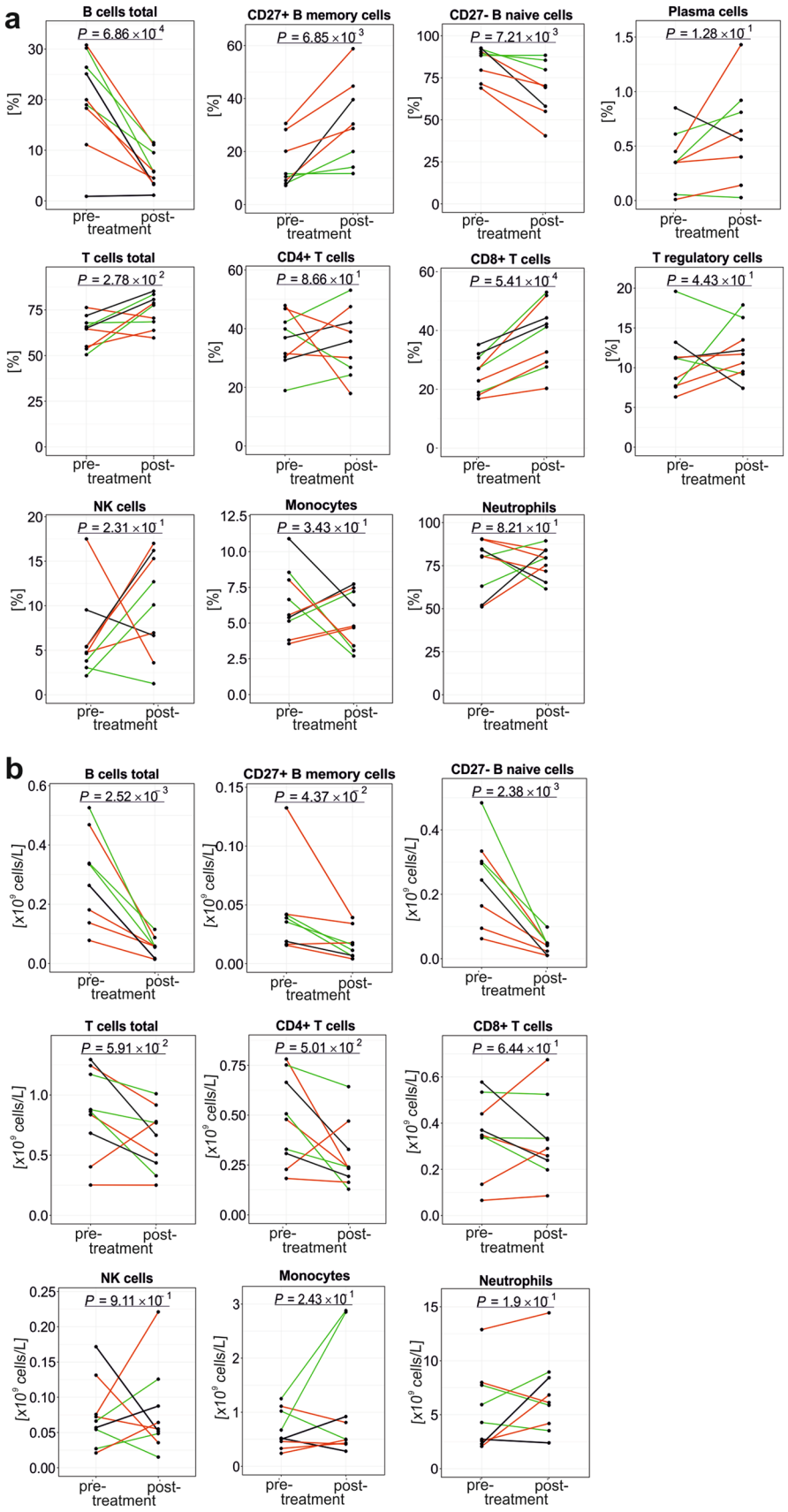

Fig. 2 Percentages (**a**) and absolute counts (**b**) of circulating immune cells in LN patients before and after the Euro-Lupus regimen treatment. In one SLE patient, $CD27^+$ B memory and $CD27^-$ B naive cells were not evaluated due to the low abundance of B cells. Patients who achieved complete remission are shown in green, partial remission in red, and non-responders in black

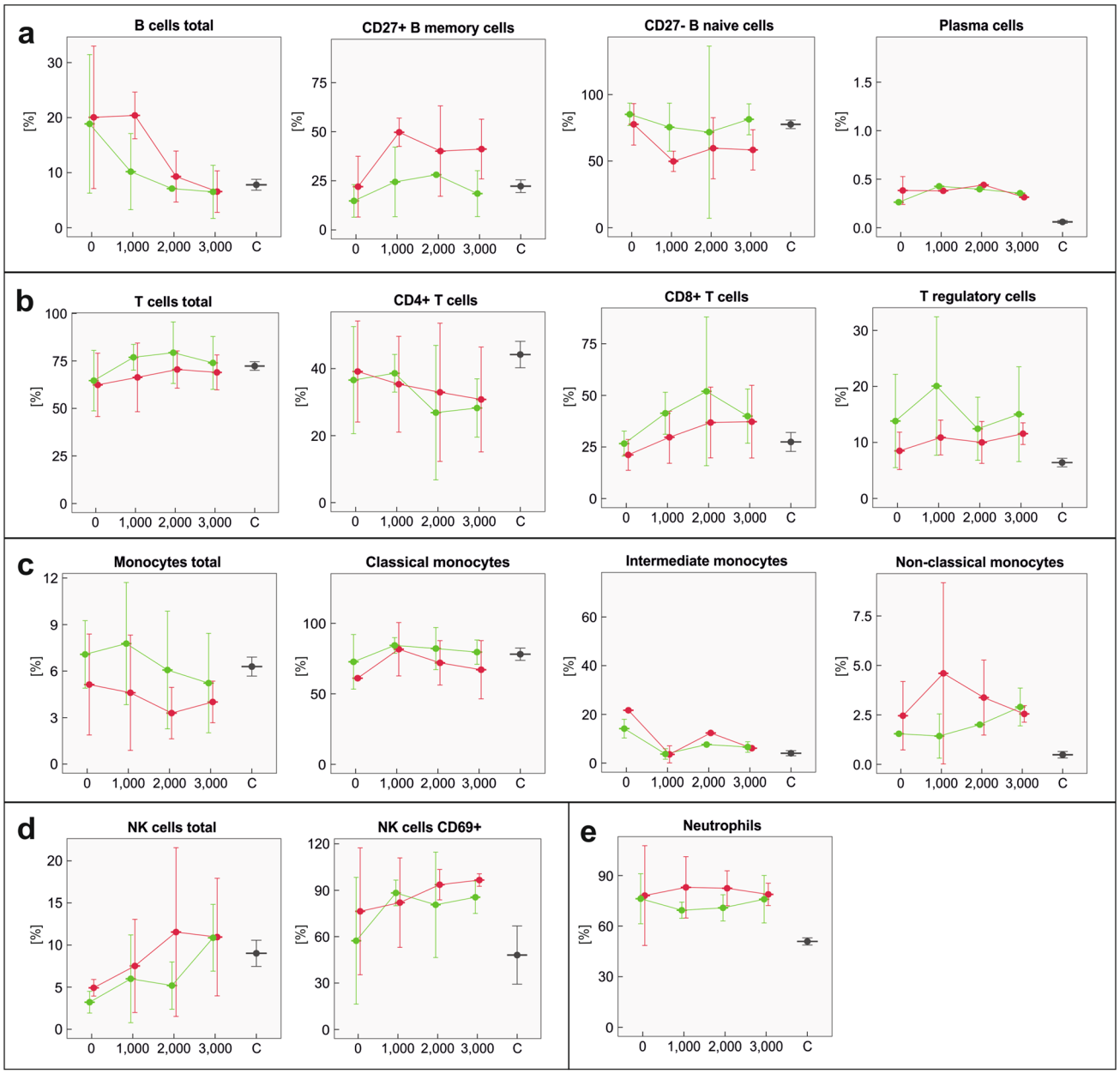

Fig. 3 Percentages of circulating immune cell populations during the Euro-Lupus regimen treatment in LN patients achieving complete remission and partial remission. **a** B cells and their subpopulations, **b** T cells and their subpopulations, **c** monocytes and their subpopulations, **d** NK cells and their activation (CD69), **e** neutrophils. Patients achieving complete remission are shown in green, patients achieving partial remission in red, and healthy controls (C) in grey. The dots represent the group means, the whiskers 95% CI. The x-axis represents the cumulative dose of CFA in mg during the Euro-Lupus regimen treatment

compared to healthy controls, showing alterations in almost all immune subpopulations and their associations with the disease activity (Alegretti et al. 2012; Boldt et al. 2014; Fassbinder et al. 2015). However, only a few studies have shown the effect of CFA on immune cells in SLE, with missing data on some populations and somewhat controversial results (Fassbinder et al. 2015; Lacki et al. 1997; Zhao et al. 2012). To our knowledge, no study has investigated whether monitoring circulating immune cells may be useful for predicting a clinical response in SLE. We therefore analysed the distributions and dynamics of immune cell populations during the Euro-Lupus low-dose intravenous CFA regimen in SLE females with various treatment outcomes. In our patients, the largest post-treatment differences were observed in B cells, which are in agreement with previous studies (Alegretti et al. 2012; Lacki et al. 1997). The

marked decrease of B cells in our patients was already evident during the first sampling point in both percentages and absolute numbers. After the Euro-Lupus regimen, most of our patients reached B cell levels comparable to those in the healthy controls, irrespective of the treatment outcome. SLE B cells play a crucial role in disease pathogenesis, as they are impaired and cannot distinguish the self from non-self-antigens (Nashi et al. 2010). They are precursors to the plasma cells that produce autoantibodies and/or proinflammatory cytokines, triggering an inflammatory response (Nashi et al. 2010). The importance of B cells in controlling the disease activity is also highlighted by the therapeutic effects of numerous B cell depletion therapies in lupus (Hui-Yuen et al. 2016). However, it has been shown that CFA-induced B cell depletion may affect the reconstitution of the B cell repertoire of SLE patients, leading to the enhanced selection of high-affinity DNA-reactive B cells, as shown in mice models (Kawabata et al. 2010).

The Euro-Lupus treatment led to marked changes in distribution of $CD27^+$ B memory cells and $CD27^-$ B naive cells in our SLE patients. The subanalysis of LN patients showed that higher proportions of $CD27^+$ B memory cells were evident particularly in patients who did not achieve CR; in CR group these cells did not exceed 20%, which is similar to the healthy controls. Regarding $CD27^+$ B memory cells, they are able to differentiate into Ig-secreting plasma blasts and have been shown to correlate with disease activity in SLE (Agematsu et al. 1998; Jacobi et al. 2008; Merrill and Buyon 2005; Odendahl et al. 2000). Whether the increase in the percentages of $CD27^+$ B memory cells in poor responders may instead be interpreted as resistance of these cells to the treatment deserves future investigations. Also, previous studies showed a modest effect of Euro-Lupus treatment on $CD27^+$ cells, but no subanalysis was performed according to the treatment outcome (Fassbinder et al. 2015). Our study suggests that $CD27^+$ B memory cell monitoring can serve as a potential early biomarker for the failure of disease treatment with CFA regimens. Future studies on larger cohorts of patients are needed to demonstrate its clinical utility.

Based on the evidence of an increased number of activated T cells and functional defects in T cell signalling in the pathogenesis of SLE (Gómez-Martín et al. 2011; Katsuyama et al. 2018; Moulton and Tsokos 2015), we also wondered about the effect on T cells, for which CFA regimens also show selectivity (Ahlmann and Hempel 2016). In agreement with a previous Euro-Lupus regimen study (Fassbinder et al. 2015), the numbers of total T cells and $CD8^+$ T cells were upregulated in our treated patients. Unlike the other report (Zhao et al. 2012), no post-treatment change in $CD4^+$ T cells was observed in our patients. $CD4^+$ and $CD8^+$ T cells, both subsets providing help to B and T regulatory cells and mediating inflammatory responses, were found compromised and aberrantly activated in the SLE patients (Moulton et al. 2017). The SLE $CD4^+$ T cells showed altered cytokine secretion with a deep defect in interleukin-2 production, and the SLE $CD8^+$ T cells showed an insufficient cytotoxic capacity (Katsuyama et al. 2018). The T cell dysfunction in SLE activates the mammalian target of rapamycin, a central regulator in cell growth, activation, proliferation, and survival not only for T cells, but also B cells and other immune, as well as non-immune, cells in SLE (Oaks et al. 2016; Perl 2016).

Regarding T regulatory cells, which play a key role in suppressing immune responses, studies on their cell numbers and function in SLE patients are contradictory (Costa et al. 2017; Horwitz 2008; Pan et al. 2012). Recent studies showed an increase in $CD45RA^+FoxP3^{low}$ T cells in peripheral blood and their defective suppressive function in vitro in SLE patients with active disease without being associated with any CFA regimen (Pan et al. 2012; Silva-Neta et al. 2018). In two of three patients achieving CR, we observed high post-treatment values of T regulatory cells. Similar results were reported in studies by Tselios et al. (2014, 2015), where the T regulatory cells were significantly expanded after the low-dose CFA treatment in LN as well as in neuropsychiatric SLE patients in which SLEDAI disease activity score was considerably reduced. Since T cells play a functional role in SLE pathogenesis, the upregulated and activated $CD8^+$ T cells and T regulatory cells, a trend evident in the responders, may contribute to better disease outcomes and must be addressed in future studies.

During the Euro-Lupus treatment, the percentage of NK cells and, namely, their activation (as assessed by an analysis of CD69) increased in the majority of patients, reaching levels higher than those in the healthy controls. Lupus NK cells have been shown to have an altered phenotype, impaired regulatory function, and low cytotoxicity, with a high expression of receptors characteristic for dendritic cells (Cruz-González et al. 2018; Park et al. 2009). Despite the upregulation of NK cells during the Euro-Lupus treatment, the similar levels of NK cells in the good and poor responders do not link the NK cells as drivers of the treatment outcome.

Finally, we wanted to describe the effect of the Euro-Lupus treatment on monocytes and neutrophils, innate cells increasingly recognised to contribute to the pathogenesis of SLE (Carmona-Rivera and Kaplan 2014; Kaplan 2011; Smith and Kaplan 2015; Wu et al. 2016).

Prior to treatment, the percentages and counts of monocytes in our SLE patients were similar to those in the healthy controls. The expression of HLA-DR on monocytes was decreased in SLE compared to the controls, which was in accordance with the literature (Shirakawa et al. 1985; Steinbach et al. 2000). The treatment in our patients did not

influence the percentage nor counts of the total monocytes, irrespective of the treatment outcome. Regarding neutrophils, almost half of our patients had neutrophilia before the initiation of the treatment, which is, in the literature, associated with progressive SLE (Lewis et al. 2016; Ren et al. 2003; Wu et al. 2016). The Euro-Lupus treatment resulted in high inter-individual variability in the distribution and counts of neutrophils in our patients and was not linked to treatment outcome. Lupus neutrophils contribute to disease pathogenesis by forming neutrophil extracellular traps, producing interferons, releasing mitochondria, diminishing phagocytic and lysosomal activity, upregulating adhesion molecules, and increasing cellular aggregation and intravascular activation in vivo (Kaplan 2011; Smith and Kaplan 2015).

This study has several limitations. First, our real-life cohort was modest and included patients with a spectrum of involved organs. Second, the effect of the Euro-Lupus low-dose intravenous CFA regimen may be caused by the joint action of CFA together with glucocorticoids and hydroxychloroquine, as all drugs are dosed simultaneously within this regimen. Despite these limitations, this proof-of-principle study shows the potential of monitoring the circulating immune cell populations in SLE patients as novel biomarkers of treatment outcomes. Future validation on a larger cohort of patients is needed.

In conclusion, these results indicate that the Euro-Lupus low-dose intravenous CFA regimen impacted several types of circulating immune cells in SLE patients, whereas changes in B cells, $CD8^+$ T cells, and their activation were predominant. Moreover, different proportions of $CD27^-$ B naive and $CD27^+$ B memory cells in groups of LN patients who achieved CR and who responded poorly (PR/NR) showed the potential of assessing immune cells for monitoring the treatment outcome, at least in Euro-Lupus regimen. A better understanding of the immunomodulatory effects of treatment regimens in patients with SLE may not only contribute to monitoring the efficacy of treatment, but also to the identification of non-responding patients and/or those who are at risk of early relapse.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no competing interests.

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