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BRIEF REPORT

Endothelial Progenitor Cell Phenotype and Function Are Impaired in Childhood-Onset Systemic Lupus Erythematosus

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Objective. **Systemic lupus erythematosus (SLE) is characterized by increased cardiovascular risk in adult-onset and childhood-onset SLE. Type I interferons (IFNs) appear to play a prominent role in premature vascular damage in adult-onset SLE, at least in part, by inducing impairments in the phenotype and function of endothelial progenitor cells (EPCs), thereby hampering vascular repair. It is not clear whether EPC dysfunction is present in childhood-onset SLE in association with a type I IFN signature.**

Methods. **The phenotype and numbers of EPCs were quantified in patients with childhood-onset SLE, patients with juvenile idiopathic arthritis (JIA), and matched healthy control subjects. In a separate cohort of patients with childhood-onset SLE, markers of subclinical atherosclerosis and endothelial dysfunction were quantified using standardized protocols and analyzed for associations with serum type I IFN activity.**

Results. **EPC numbers and function were significantly decreased in patients with childhood-onset SLE compared with patients with JIA and healthy control subjects. Serum from patients with childhood-onset SLE impaired differentiation of EPCs into mature endothelial cells in healthy controls, and this effect was blocked by inhibition of the type I IFN pathway. Type I IFN activity in serum was not significantly associated with subclinical atherosclerosis and endothelial function in patients with childhood-onset SLE.**

Conclusion. **As in adult-onset SLE, childhood-onset SLE is characterized by phenotypic and functional EPC abnormalities, which are likely triggered by type I IFNs. Although cross-sectional analysis revealed no global association between type I IFN signatures and vascular measures of subclinical atherosclerosis, longitudinal assessments are needed to evaluate whether progression of vascular damage in patients with childhood-onset SLE is associated with type I IFNs, as observed in patients with adult-onset SLE.**

Cardiovascular (CV) morbidity and mortality are increased in patients with systemic lupus erythematosus (SLE) and cannot be explained using the Framingham risk equation (1,2). Patients with childhood-onset SLE have a greater disease burden due to a younger age at onset and often present with more severe clinical manifestations and major organ involvement (3). Subclinical atherosclerosis has been reported in childhood-onset SLE, with significant increases in carotid intima-media thickness (CIMT) (4,5). Although treatment with atorvastatin may decrease the progression of CIMT in a subset of patients with childhood-onset SLE, it did not reduce the overall atherosclerosis risk (6), indicating that other factors involved in the pathophysiology of endothelial damage play a central role in vascular pathology.

We and other investigators have proposed that type I interferons (IFNs) play an important role in the pathogenesis of accelerated atherosclerosis in patients with adult-onset SLE (7–9), through deleterious pleiotropic effects on the vasculature. Type I IFNs induce significant impairment of the capacity of endothelial progenitor cells (EPCs) to differentiate into mature endothelial cells (ECs) and repair the vasculature (7,10).

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In murine models of lupus and atherosclerosis, type I IFN blockade abrogates endothelial dysfunction, the prothrombotic phenotype, plaque formation, and abnormal vasculogenesis (11,12). We previously showed associations between type I IFN activity in serum with vascular dysfunction and enhanced subclinical carotid and coronary atherosclerosis in patients with adult-onset SLE (13). These observations suggest a crucial role for these cytokines in initiating and perpetuating atherosclerotic lesions in patients with SLE.

A strong inverse correlation between CV risk factors and the number and function of circulating EPCs in adults with various CV risk factors was previously reported (7,10). Although little is known about EPCs and their role in atherogenesis in children, there is evidence that certain pediatric populations at risk of vascular disease have impaired EPC phenotype and function that correlate with endothelial dysfunction (14,15).

We hypothesized that childhood-onset SLE is also characterized by aberrant EPC phenotype and function secondary to enhanced type I IFN activity. We posit that these abnormalities may promote endothelial dysfunction and accelerated vascular damage in children. We analyzed EPC phenotype and function in patients with childhood-onset SLE and the association with type I IFN activity. We also assessed the association between type I IFN activity and vascular measures of early atherosclerosis.

PATIENTS AND METHODS

Subject recruitment. Age- and sex-matched healthy control subjects and patients with childhood-onset SLE fulfilling the American College of Rheumatology criteria (16) were enrolled at the Hospital for Sick Children (HSC; 132 patients and 170 healthy controls) and at the University of Michigan (UM; 25 patients and 29 healthy controls). Children fulfilling the International League Against Rheumatism criteria for juvenile idiopathic arthritis (JIA; n = 21) (17) were enrolled at UM. In the HSC cohort of patients with SLE, serum was obtained for assessment of type I IFN activity, and subclinical atherosclerosis and endothelial dysfunction were quantified by CIMT using a GE Vivid 7 ultrasound system (GE Vingmed), a 12-MHz linear array transducer, and a Vascular Tools Carotid Analyzer (Medical Imaging Applications LLC); by brachial artery flow-mediated dilation (FMD), using the same ultrasound system and a Vascular Tools Brachial Analyzer (Medical Imaging Applications LLC); and by pulse wave velocity (PWV), using a Millar SPC-301 high-fidelity micromanometer and a SphygmoCor system (AtCor Medical). Vascular measures were compared with normative data acquired using the same standardized protocols (4). Institutional review boards approved the study, and all subjects provided written informed consent.

EPC quantification. In the UM cohorts of healthy control subjects, patients with SLE, and patients with JIA, peripheral blood mononuclear cells (PBMCs) were obtained, and EPCs were quantified by fluorescence-activated cell sorting analysis as previously described (7,8,11–13), on a FACSCalibur flow cytometer (BD Biosciences). A total of 10,000 events were quantified, and the results were analyzed using FlowJo software. EPCs were characterized as described by our group (CD34+CD133+CD4−CD3−CD56−CD79b−).

Assessment of EC differentiation. PBMCs were plated on fibronectin-coated plates (4 million cells/well; BD Biosciences) and cultured for 2 weeks in endothelial growth medium with 20% fetal bovine serum (FBS) (Gibco BRL) or 20% allogeneic serum from healthy control subjects, patients with JIA, or patients with childhood-onset SLE. The media was changed every 3 days. After 14 days, differentiation into mature ECs was quantified by assessing coexpression of Texas red–conjugated acetylated low-density lipoprotein (Biomedical Technologies) and fluorescein isothiocyanate–conjugated *Ulex europaeus* agglutinin type I (Vector) by fluorescence microscopy (Leica DMIRB inverted microscope) using Cell C cell-counting software (12). Seven photomicrographs were obtained per field, with results reported as the mean ± SEM per sample. For studies using allogeneic human serum, cells were cultured in the presence or absence of 2 μg/ml neutralizing anti-human interferon α/β (IFNα/β) receptor (PBL Biomedical) or IgG2a isotype (Abcam). Antibody was added at each media change every 2–3 days, and cells were quantified as described above.

Serum type I IFN activity. This bioassay has been described elsewhere (13). Briefly, HeLa cells were incubated with Dulbecco's modified Eagle's medium/10% FBS (negative control), 1 Kunit/well recombinant IFNα (Invitrogen) as positive control, 50% SLE sera (by volume), or 50% control sera for 6 hours. RNA was extracted using RNeasy (Qiagen) and reverse transcribed to complementary DNA (Invitrogen). Real-time polymerase chain reaction was performed in triplicate on an ABI Prism 7900HT system (Applied Biosystems) using 2× SYBR Green Supermix (Bio-Rad), to quantify the following 5 type I IFN-inducible genes (IFIGs): myxovirus resistance protein 1 (*MX1*), double-stranded RNA activated protein kinase (*PRKR*), IFN-induced protein with tetratricopeptide repeats (*IFIT1*), IFN-induced protein 44 (*IFI44*), and IFN-induced protein 44-like (*C1orf29*); the housekeeping gene hypoxanthine guanine phosphoribosyltransferase 1 (*HPRT1*) was also quantified. Primers were obtained from Integrated DNA Technologies. The results of quantification were averaged, normalized to the housekeeping gene, and plotted as the fold induction relative to 25 age- and sex-matched healthy control subjects.

Statistical analysis. Multivariable linear regression analyses were performed to determine associations between individual IFIGs and vascular markers (CIMT, FMD, and PWV), adjusting for age, sex, and medications. A principal components analysis was applied to assess for differences in the profiles of IFIGs as compared with vascular measures, as described for adult-onset SLE (13). Differences in mean values for EPC quantification and differentiation were analyzed using Student's *t*-test. Clinical data were adjusted for disease activity. To examine associations between clinical and demographic variables and vascular dysfunction, a Mann-Whitney test comparing medians was used for categorical data, because outcomes were not normally distributed, and simple linear regression was used for continuous variables. Differences in the demographic data of the cohorts were assessed for significance using chi-

Table 1. Demographic and clinical characteristics of the study subjects*

	Hospital for Sick Children		University of Michigan			
	Childhood-onset SLE (n = 132)	Healthy controls (n = 178)	Childhood-onset SLE (n = 25)	JIA (n = 21)	Healthy controls (n = 29)	P†
Age, mean ± SEM years	14.8 ± 2.7	14.4 ± 2.1	18.2 ± 2.7	15.4 ± 2.3	14.5 ± 4.7	<0.001‡
No. male/no. female	23/109	84/94	3/22	8/13	8/21	0.078§
Disease duration, mean ± SEM years	2.1 ± 2.1	NA	4.0 ± 3.0	6.0 ± 5.4	NA	<0.01¶
Disease duration range, years	0.2−13.3	NA	0−9.0	0−15.0	NA	–
Current medications						
NSAIDs	14 (11)	–	10 (40)	16 (76)	–	<0.001
ACE inhibitors or ARBs	25 (19)	–	15 (60)	None	–	<0.001
Prednisone	82 (62)	–	13 (52)	1 (5)	–	0.377
Hydroxychloroquine	114 (86)	–	24 (96)	12 (57)	–	0.008
Mycophenolate	24 (18)	–	11 (44)	None	–	0.008
Azathioprine	42 (32)	–	3 (12)	None	–	0.054
Cyclophosphamide	17 (13)	–	1 (4)	None	–	0.312
Methotrexate	7 (5)	–	1 (4)	5 (24)	–	0.008
Cyclosporine	3 (2)	–	2 (8)	1 (5)	–	0.323
IVIG	4 (3)	–	0 (0)	None	–	1
Vascular markers						
CIMT, mean ± SEM mm	0.410 ± 0.050	0.439 ± 0.045	–	–	–	<0.001
FMD, mean ± SEM % change	8.2 ± 3.9	7.5 ± 3.2	–	–	–	0.808
PWV, mean ± SEM mm/second	5.4 ± 1.0	5.1 ± 0.9	–	–	–	0.328

* Medications that are used primarily to treat systemic lupus erythematosus (SLE) were compared only between the 2 cohorts of patients with childhood-onset SLE, because these medications are not used to treat juvenile idiopathic arthritis (JIA). Except where indicated otherwise, values are the number (%). NA = not applicable; NSAIDs = nonsteroidal antiinflammatory drugs; ACE = angiotensin-converting enzyme; ARBs = angiotensin II receptor blockers; IVIG = intravenous immunoglobulin; CIMT = carotid intima-media thickness; FMD = flow-mediated dilation; PWV = pulse wave velocity.

† By one-way analysis of variance for multiple comparisons of continuous variables and chi-square or Fisher's exact test for multiple comparisons of categorical variables.

‡ Hospital for Sick Children SLE patients versus University of Michigan SLE patients; other age differences were not significant.

§ By chi-square test.

¶ Hospital for Sick Children SLE patients versus University of Michigan SLE patients.

square or Fisher's exact tests for categorical variables and one-way analysis of variance for continuous variables.

RESULTS

Patient characteristics. The demographic and clinical characteristics of the patients with childhood-onset SLE, the patients with JIA, and the healthy control subjects are shown in Table 1. Patients at HSC were younger than patients at UM, because the minimum ages at the time of enrollment were 9 years and 12 years, respectively, due to the large volume of blood needed for EPC experiments. Disease duration was longer in the UM patients compared with the HSC patients. The mean disease duration was longer in the UM patients with childhood-onset SLE compared with the HSC patients with childhood-onset SLE, and in the UM cohort of patients with JIA, disease was more long-standing at the time of enrollment than in patients with childhood-onset SLE. Most patients with childhood-onset SLE were receiving hydroxychloroquine, half were receiving a steroid-sparing agent, and most of those receiving prednisone were receiving <0.5 mg/kg daily.

Vascular testing. The mean ± SEM CIMT in healthy controls was 0.439 ± 0.045 mm and was significantly higher that in patients with childhood-onset SLE (0.410 ± 0.050 mm; $P < 0.001$). FMD values were higher in patients with childhood-onset SLE compared with healthy control subjects (mean ± SEM 8.2 ± 3.9% versus 7.5 ± 3.2%), but the difference was not statistically significant ($P = 0.808$). PWV values assessing arterial stiffness did not differ between patients with childhood-onset SLE and healthy control subjects (mean ± SEM 5.4 ± 1.0 mm/second versus 5.1 ± 0.9 mm/second; $P = 0.328$) (Table 1). Less than 2% of patients with childhood-onset SLE undergoing vascular testing had results that deviated >2 SD from those in healthy control subjects (for CIMT, for 4 of 127 SLE patients; for PWV, for 4 of 126 SLE patients; for FMD, for 0 of 127 SLE patients). As such, the baseline data of the childhood-onset SLE cohort did not suggest significant vascular dysfunction in these patients.

Effect of type I IFNs on phenotype and function of EPCs in patients with childhood-onset SLE. The number of circulating EPCs was significantly decreased

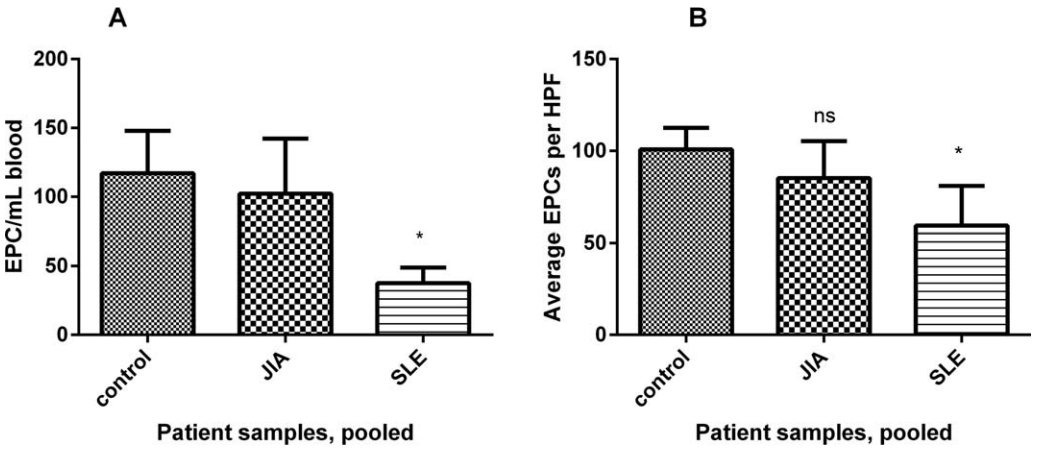

Figure 1. Numbers and differentiation of endothelial progenitor cells (EPCs) in healthy control subjects, patients with juvenile idiopathic arthritis (JIA), and patients with childhood-onset systemic lupus erythematosus (SLE). **A,** Numbers of EPCs in the circulating blood of healthy control subjects (n = 14), patients with JIA (n = 9), and patients with childhood-onset SLE (n = 19). **B,** EPC differentiation in healthy control subjects, patients with JIA, and patients with childhood-onset SLE, as assessed by fluorescence microscopy (n = 12 per group). Coexpression of Texas Red–conjugated acetylated low-density lipoprotein and fluorescein isothiocyanate–conjugated *Ulex europaeus* agglutinin type I identified mature ECs. Values are the mean ± SEM. hpf = high-power field; NS = not significant. * = $P < 0.05$ versus control and JIA.

in patients with childhood-onset SLE compared with the numbers in healthy control subjects and patients with JIA (Figure 1A), and no correlation with age ($P = 0.391$), disease duration ($P = 0.692$), or immunosuppressive medications (for mycophenolate, $P = 0.062$; for prednisone, $P = 0.442$) was observed. In comparison with EPCs from healthy control subjects or patients with JIA, EPCs from patients with childhood-onset SLE displayed a significantly decreased capacity to differentiate into mature ECs ($P = 0.01$) (Figure 1B). Increasing age appeared to be associated with decreased differentiation, as determined by linear regression analysis ($P = 0.034$), while disease duration was not associated with decreased differentiation ($P = 0.242$). There was no association between treatment with mycophenolate or azathioprine and the number or function of EPCs ($P = 0.399$ and $P = 0.167$, respectively) when comparing patients receiving these drugs and those not receiving these drugs. Insufficient numbers of patients were receiving high-dose prednisone (≥1 mg/kg/day) or cyclophosphamide to allow testing for changes in EPC function. There was no correlation between EPC levels in patients with childhood-onset SLE and the Systemic Lupus Erythematosus Disease Activity Index (18), body mass index (BMI), systolic blood pressure, the creatinine level, the erythrocyte sedimentation rate (ESR) the C-reactive protein (CRP) level, or the lipid profile ($P > 0.05$).

To assess the role of circulating type I IFNs in inhibiting EC differentiation in patients with childhood-onset SLE, EPCs obtained from healthy control subjects were differentiated into mature ECs in 20% allogeneic serum from healthy control subjects, patients with JIA, and patients with childhood-onset SLE, under proangiogenic stimulation (Figure 2A), as previously described (12). Serum from patients with childhood-onset SLE, but not that from patients with JIA, significantly hampered EC differentiation when compared with serum from healthy control subjects ($P < 0.01$) (Figure 2B). The detrimental effect of serum from patients with childhood-onset SLE on EC differentiation was significantly attenuated by neutralizing antibodies to IFNα/β receptor ($P < 0.05$) (Figure 2C). Neutralizing antibodies to IFNα/β receptor had no significant effect on control EPCs that were exposed to serum from patients with JIA or serum from healthy control subjects.

Type I IFN signatures and vascular assessments in childhood-onset SLE. Serum type I IFN activity was significantly increased in patients with childhood-onset SLE (additional information is available from the corresponding author). Serum IFN activity did not correlate with the body mass index, systolic blood pressure, the creatinine level, the CRP level, the ESR, or lipid profiles. Post hoc analysis showed a trend toward an association between higher expression of the IFN-inducible genes *PRKR* and *C1orf29* in those patients with childhood-onset SLE who had CIMT values that deviated >1 SD from the mean values in healthy control subjects ($P = 0.053$ and $P = 0.074$, respectively).

DISCUSSION

Type I IFNs appear to play important roles in SLE pathogenesis and in the accelerated atherosclerosis characteristic of this disease (8,9,11–13). In adult-onset

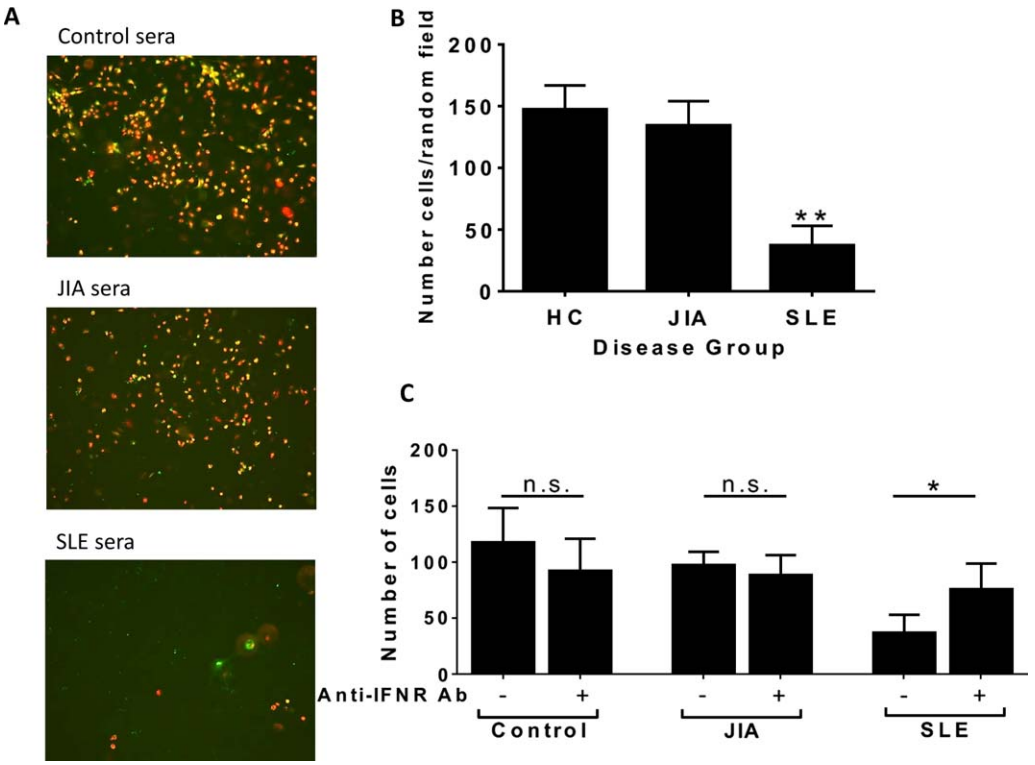

Figure 2. Role of circulating interferon-α (IFNα) on EPCs in childhood-onset SLE. **A,** Representative photomicrographs of EPCs from healthy control (HC) subjects cultured for 14 days in 20% sera from healthy control subjects (n = 5), patients with JIA (n = 5), and patients with childhood-onset SLE (n = 7). Cells were labeled with DiI-labeled acetylated low-density lipoprotein (red) and fluorescein isothiocyanate–conjugated *Ulex europaeus* agglutinin type I (green). Mature endothelial cells coexpressed both markers (yellow). Original magnification $\times$ 10. **B,** Effect of sera from healthy control subjects, patients with JIA, and patients with childhood-onset SLE on the survival of healthy EPCs on day 15. ** = $P < 0.01$ versus control, by one-way analysis of variance followed by Dunnett's multiple comparison test. **C,** Effect of IFNα signaling blockade on EC differentiation in control cells exposed to sera from healthy control subjects, patients with JIA, and patients with SLE. * = $P < 0.05$, by paired *t*-test. Values in **B** and **C** are the mean $\pm$ SEM. See Figure 1 for other definitions.

SLE, type I IFN activity has been linked to decreased vascular function, higher CIMT, and coronary calcification (13). Murine and human studies have shown that type I IFNs have significant pleiotropic effects that are deleterious to the vasculature, including the promotion of endothelial damage, impairment of endothelial repair, facilitation of foam cell formation, and enhancement of thrombosis (11,12). Indeed, type I IFNs are directly cytotoxic to EPCs and impair their function (7,10,14).

It is well recognized that childhood-onset SLE is associated with an elevated type I IFN signature (9); however, it is unknown whether children are equally susceptible to EPC impairment and accelerated vascular damage by type I IFNs. Decreased numbers of EPCs and impaired endothelial function have been described in other pediatric autoimmune diseases, such as type 1 diabetes mellitus (15), and obese children display decreased numbers of circulating EPCs in association with vascular dysfunction (14). We observed decreased numbers and function of circulating EPCs from patients with childhood-onset SLE, similar to what we and other investigators observed in adult-onset SLE. Increasing age was the only variable that significantly correlated with reduced EPC differentiation. Immunosuppression did not appear to impact EPC differentiation, although too few patients were receiving high-dose prednisone or cyclophosphamide to allow analysis of these contributions.

Previous studies in patients with adult-onset SLE showed that IFIGs were associated with decreased endothelial function, increased CIMT, arterial stiffness, and the severity of coronary calcification (13). We could not confirm these observations in patients with childhood-onset SLE, even though the levels of type I IFNs were increased in these patients compared with healthy controls, and CIMT was in fact lower in patients with childhood-onset SLE compared with healthy controls. The lack of significant differences in vascular measurements between patients with childhood-onset SLE and healthy controls is likely a main factor in the lack of correlation with type I IFNs. Indeed, only a minority of

patients with childhood-onset SLE had abnormal values, unlike those observed in the study of adult patients. Furthermore, a post hoc exploratory analysis showed a trend toward an association of specific IFIGs and higher CIMT values. The significance of these findings needs to be confirmed in an independent cohort in which more subjects have abnormal values. As such, the differences between the adult and pediatric SLE studies regarding associations between type I IFN responses and functional/anatomic evidence of cardiovascular disease (CVD) are likely related to a lack of prominent vascular damage in this cohort of patients with childhood-onset SLE. Longer followup will be needed to assess the role of these cytokines in the progression of CVD in childhood-onset SLE.

The results of this study suggest that, similar to adult-onset SLE, childhood-onset SLE is characterized by impaired EPC phenotype and function, which are likely driven by type I IFNs. Future longitudinal studies should systematically assess whether these abnormalities contribute to the severity and/or progression of premature vascular damage and CV events in childhood-onset SLE.

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AUTHOR CONTRIBUTIONS

All authors were involved in drafting the article or revising it critically for important intellectual content, and all authors approved the final version to be published. Dr. Kaplan had full access to all of the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

Study conception and design. Barsalou, Bradley, Kaplan.

Acquisition of data. Mohan, Barsalou, Bradley, Slorach, Reynolds, Hasni, Thompson, Ng, Levy, Silverman.

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