Systemic lupus erythematosus, endothelial progenitor cells and intracellular Ca^{2+} signaling: A novel approach for an old disease

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ABSTRACT

Systemic lupus erythematosus (SLE) is an autoimmune multisystem disease featured by an increased cardiovascular risk that may lead to premature patient's death. It has been demonstrated that SLE patients suffer from early onset endothelial dysfunction which is due to the impairment of endogenous vascular repair mechanisms. Vascular integrity and homeostasis are maintained by endothelial progenitor cells (EPCs), which are mobilized in response to endothelial injury to replace damaged endothelial cells. Two main EPCs subpopulations exist in peripheral blood: endothelial colony forming cells (ECFCs), which represent truly endothelial precursors and can physically engraft within neovessels, and myeloid angiogenic cells (MACs), which sustain angiogenesis in a paracrine manner. Emerging evidence indicates that ECFCs/MACs are down-regulated and display compromised angiogenic activity in SLE, thereby contributing to the pathogenesis of this disease. Intracellular calcium (Ca^{2+}) signaling plays a crucial role in maintaining vascular integrity by stimulating migration, proliferation and tube formation in both ECFCs and MACs. Herein, we illustrate the evidences that support the role played by EPCs dysfunction in SLE. Subsequently, we discuss about the hypothesis that the Ca^{2+} handling machinery is compromised in SLE-derived ECFCs and MACs, thereby resulting in their reduced pro-angiogenic activity. Finally, we speculate about the proposal to exploit intracellular Ca^{2+} signaling to improve ECFCs' reparative phenotype and suggest this strategy as a new approach to treat SLE patients.

1. Introduction

Systemic Lupus Erythematosus (SLE) has been intensively studied and is described as a multisystem autoimmune disorder with serological and clinical heterogeneity that primarily affects young women in their childbearing age [1–3]. SLE is associated with a fivefold increased risk of cardiovascular disease attributed to the high prevalence of hypertension [4], dyslipidemia [5], diabetes [6], and smoking [7]. Independently on traditional cardiovascular risk factors, SLE is further characterized by an early onset of endothelial dysfunction related to chronic inflammation and oxidative stress [4,6,8–12]. Endothelial cells play a fundamental role in the regulation of vascular tone and

permeability, platelet aggregation and coagulation, inflammation and angiogenesis [9,13–15]. Endothelial damage is triggered by binding of autoantibodies and immune complex to endothelial tissue, which leads to increased pro-coagulation, anti-fibrinolytic activity, vasoconstriction and atherosclerosis development [9,10,16–21]. Preservation of endothelial integrity by maintaining the equilibrium between endothelial damage and repair is of crucial importance. Endothelial progenitor cells (EPCs) are intimately involved in the compensatory response to endothelial dysfunction by their ability to differentiate into mature endothelial cells and maintaining vascular integrity. Endothelial colony forming cells (ECFCs) and myeloid angiogenic cells (MACs) are well-defined and distinct cellular populations that may fall within the

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definition of EPCs [22–25]. The reduced number of MACs in peripheral blood has been associated with increased risk of atherosclerosis and cardiovascular disease [26–28]. Although atherosclerosis appears to share a pathogenic pathway with SLE, available data about MACs frequency in different stages of SLE bring contrasting results. However, it has been reported that MACs are characterized by defective mobilization and regenerative ability in SLE patients, which hints at their contribution to the pathophysiology of SLE [29]. Likewise, ECFCs are down-regulated and display reduced pro-angiogenic activity in individuals affected by cardiovascular disorders [30–32]. Furthermore, their regenerative ability is compromised in SLE [33,34]. In this review, we first discuss the origin, classification and function of EPCs. Then, we describe the endothelial dysfunction and illustrate the evidences that support the role played by ECFCs and MACs in SLE. Subsequently, we discuss about the hypothesis that the Ca^{2+} handling machinery is compromised in SLE-derived MACs and ECFCs, thereby resulting in their reduced pro-angiogenic activity. Finally, we speculate about the proposal to exploit intracellular Ca^{2+} signaling to improve ECFCs' reparative phenotype and suggest this strategy as a new approach to treat SLE patients.

1.1. Endothelial progenitor cells: origin, classification and function

EPCs represent a heterogeneous group of cells mobilized from either bone marrow or vascular stem cell niches, which contribute to endothelial repair, vascular homeostasis and neovascularization [22,24,35,36]. Over the past 20 years, several methods have been widely used to identify and characterize EPCs from peripheral and umbilical cord blood. These include: culture and colony forming assays from mononuclear cells (MNCs), flow cytometry, cell sorting and functional angiogenic assays (e.g. tube formation in Matrigel scaffolds and/or development of perfused vascular networks *in vivo*) [22,35]. Each of these methods may present some limitations, including cell culture contamination with false positive cells, absence of unique antigens and highly specific markers to identify truly endothelial precursors, and lack of selective molecular signatures [22,37,38]. It has been suggested that single-cell transcriptomics and proteomics could help resolve the major issue of identifying a phenotypic marker restricted to a specific stem or progenitor cell population [35]. Even though a “universal” methodology for the identification and differentiation of EPCs is still lacking, the combination of phenotypic, functional, morphologic, and “omics” information has been employed to identify two distinct subtypes of circulating EPCs, as illustrated in a recent position paper [22]: 1) myeloid angiogenic cells (MACs) and 2) endothelial colony forming cells (ECFCs).

MACs comprise multiple cellular populations belonging to the hematopoietic lineage, including circulating angiogenic cells (CACs), early EPCs, colony forming unit (CFU)-Hill (CFU-Hill) EPCs, CFU-endothelial cells (CFU-ECs), and hematopoietic EPCs [22]. MACs derive from MNCs harvested from both peripheral and umbilical cord blood and cultured under endothelial cell culture conditions [22]. They display an immune phenotype positive for CD34, CD45, CD14, and CD31, while they are negative for CD146 [22]. MACs do not differentiate into endothelial cells, but promote angiogenesis and vasoreparation through paracrine signaling [22,36]. Nevertheless, CFU-ECs, as well as CACs, sustain angiogenesis by secreting paracrine signals that stimulate endothelial cells to sprout from adjoining capillaries and recruit circulating ECFCs to nascent vasculature [22,35].

ECFC colonies emerge 10–21 days after plating MNCs on type I rat tail collagen-coated dishes in the presence of an endothelial growth medium and are, therefore, also known as late EPCs or blood outgrowth endothelial cells (BOECs). ECFCs display the cobblestone morphology typical of vascular endothelial cells, form capillary-like structures when plated in Matrigel scaffolds and anastomose with host vasculature *in vivo* [35,39,40]. Consistently, ECFCs are now regarded as “*bona fide*” endothelial precursors [22,35] and were shown to maintain their

endothelial phenotype over a prolonged period in culture [41]. ECFCs present significant proliferative and clonogenic potential, with some ECFC colonies, known as high proliferative potential ECFCs (HPP-ECFCs), being able to give rise to secondary and tertiary colonies on replating [39,40]. ECFCs do not express a cell surface panel of novel specific antigens, but are negative for the hematopoietic markers CD45, CD14, CD115, and AC133. Conversely, ECFCs present a number of markers that can also be found in vascular endothelial cells, such as VEGFR2, CD31, CD34, CD105, CD144, CD146, VE-cadherin and von Willebrand factor [22,40,42]. Furthermore, ECFCs show additional endothelial markers, including acetylated low-density lipoprotein (Ac-LDL) uptake and *Ulex europaeus* agglutinin 1 (UEA-1) binding [42]. Besides peripheral and umbilical cord blood [40], human ECFCs can be harvested from tissue-resident vascular endothelium, including aorta [39], lung tissue [43], pulmonary artery [44], saphenous vein [45], placenta [46], or can be obtained from white-adipose tissue [47] or from induced pluripotent stem cells (iPSCs) [48]. Nevertheless, the exact origin of circulating ECFCs remains unclear. Emerging body of evidence hinted at the existence of vascular resident endothelial cells that possess clonogenic ability and are able to induce neovessel formation *in vivo* [49–51]. However, transcriptomic analysis revealed that circulating ECFCs are closer to microvascular, rather than macrovascular, endothelial cells [52]. Whatever their precise source along the vascular tree, ECFCs represent the most suitable cell substrate for therapeutic angiogenesis of ischemic diseases [35,53–55]. For instance, myocardial injection of ECFCs increased microvascular density, ameliorated maladaptive remodeling and improved cardiac function in rat [56], mouse [57], and porcine [58] models of acute myocardial ischemia (AMI). Likewise, ECFCs delivery promoted neovascularization and boosted local blood flow recovery in murine models of hindlimb [59–61] and retinal ischemia [62], as well as in traumatic brain injury [63,64] and ischemic stroke [65,66]. ECFCs may exploit multiple mechanisms to increase microvascular density in damaged tissues, which makes them the most suitable tool for regenerative purposes: 1) direct incorporation into nascent neovessels [41,67]; 2) paracrine release of pro-angiogenic growth factors and cytokines, trophic factors or exosomes [68–70]; and 3) support of the regenerative potential of other stem cell types, including mesenchymal progenitor cells [59,71] and adipose stromal cells [72]. It has been proposed that ECFCs cooperate with MACs to regenerate damaged vascular networks. MACs were predicted to represent the first EPC subset to be targeted to the injury site [36], thereby liberating a host of growth factors and chemokines which recruit circulating ECFCs from blood stream or the adjacent vascular stem cell niche. Once reached nascent neovasculature, ECFCs proliferate, migrate, incorporate into vascular wall and/or release pro-angiogenic cues [35,39].

2. Endothelial injury in systemic lupus erythematosus: the failure of endothelial repair mechanisms

SLE is featured by raised mortality rate secondary to cardiovascular disorders, which account for one-third of deaths among SLE patients [5]. For instance, the prevalence of vascular ischemic events, such as AMI and brain stroke, is equal to approximately 6–10% in SLE patients and is responsible for 20–30% of SLE deaths [73]. Traditional risk factors (e.g. hypertension, dyslipidemia, diabetes and smoking) are not enough to explain the exceedingly high rate of cardiovascular complications reported in SLE patients. Indeed, although the incidence of cardiovascular diseases has long been known to increase in the aging population, the incidence of AMI, congestive heart failure and cerebrovascular accident is significantly enhanced in young women affected by SLE [5,74]. More in general, SLE patients present an accelerated rate of plaque progression that predisposes them to undergo coronary artery disease and stroke earlier than sex- and age-matched controls [5,75]. Notably, premature atherosclerosis in SLE patients is quite atypical as compared to “idiopathic” atherosclerosis as it is not associated to the

classical inflammatory burden caused by increased plasma levels of low density lipoprotein (LDL) and elevated C-reactive protein (CRP) [5]. It may, therefore, be concluded that, after correcting for all traditional Framingham risk factors, SLE operates as an independent atherogenic risk factor [75].

2.1. The causative mechanisms of endothelial injury in SLE

Endothelial injury has long been regarded as one of the earliest events in the pathogenesis of atherosclerosis, playing a crucial role in plaque initiation and progression [9,76,77]. Likewise, it has been documented that loss of endothelial integrity is indispensable to promote the dramatic alterations in the arterial wall that result in the premature onset of atherosclerosis in young SLE patients [9,10,78,79]. Endothelial dysfunction impairs the control of vascular tone and permeability even before atherosclerotic lesions become detectable in SLE patients [10,80]. For instance, endothelium-dependent vasodilation, also termed flow-dependent vasodilation (FMD), is significantly reduced in SLE patients due to the impairment in nitric oxide (NO) release [9,81,82]. Additional surrogates of endothelial dysfunction, such as increased carotid artery intima thickness and coronary artery calcifications, represent a typical clinical feature of SLE [10,83].

Apart from the traditional cardiovascular risk factors, endothelial injury in SLE is accelerated by the chronic inflammatory state, immune system activation and oxidative stress [10]. As a chronic autoimmune disease involving multiple organs, SLE is characterized by a lower clearance of apoptotic cells and immune complexes (ICs) and by the loss of immunological tolerance of B cells, which release in circulation a bewildering amount (up to 180) of autoantibodies directed against multiple targets, including double-stranded DNA, nuclear proteins, ribonuclear proteins, such as Ro, La and Sm, phospholipids, and annexin V [18,84–87]. Endothelial damage is, therefore, initially triggered by the deposition of this array of autoantibodies and ICs on the intimal layer of the vasculature. Of note, both anti-DNA and non-anti-DNA antibodies, also known as anti-endothelial cell autoantibodies (AECAs), derived from SLE sera induce apoptosis in cultured vascular endothelial cells by recognizing endothelial antigens [88,89]. For instance, AECAs may induce endothelial cell death by binding to the 60 kD heat-shock protein (Hsp60) [90], the COOH-terminal of Ral binding protein1 (RLIP76) [91], and rabaptin 5 [92]. Additional endothelial targets of the AECAs contained in SLE sera and responsible for endothelial cell damage sera remain to be identified [89]. Likewise, endothelial cell apoptosis may be induced by SLE anticoagulant [93,94] and by anti-double-stranded DNA antibodies [95]. In addition, vascular endothelial cells may be injured following exposition to microparticles (MPs) [96], which are increased and form ICs in SLE patients [97], and by neutrophil associated traps (NETs), whose formation increases in SLE and results in a cell death mode which exhibits distinct features as compared to apoptosis and necrosis [98]. A further source of endothelial dysfunction in SLE patients is provided by antiphospholipid antibodies, including cardiolipin, anti- β_2 -glycoprotein I (anti- β_2 GPI), the lipid-associated enzyme lipoprotein lipase (LPL) and the high-density lipoprotein (HDL) component apolipoprotein A-I (Apo A-I) [80,99]. The complement component C1q, which binds to negatively charged and polyanionic targets, such as phosphatidyl serine and DNA, respectively, plays a crucial role in clearing apoptotic cells and preventing local and systemic inflammation [100]. Furthermore, it has recently been demonstrated that C1q contributes to maintain vascular integrity by promoting angiogenesis and by mediating the interaction between endothelial cells and extracellular matrix [100]. It has, therefore, been proposed that the reduction or absence of C1q due to either genetic or acquired factors, could be involved in SLE development [81,100]. Finally, a recent investigation revealed that the complement component C5a is also involved in endothelial cell apoptosis by activating the caspase cascade via the selective C5a receptor 1 (C5aR1) in a mouse model of SLE [101].

2.2. Endogenous mechanisms of endothelial repair are compromised in SLE

Endothelial damage could be counteracted by endothelial regeneration upon recruitment of resident ECFCs [26,39,50,51], while bone marrow-derived MACs could sustain the reparative process in a paracrine manner [36,102]. However, endothelial repair is severely compromised by SLE, thereby boosting endothelial dysfunction and resulting in premature vascular damage [5,8,26,34,103]. The main culprit of the imbalance between endothelial injury and vascular repair is represented by type 1 interferons (IFNs) [5,8,26,103]. Type 1 IFNs comprise an array of structurally related cytokines that contribute to establish the immune response against viral infections by acting through type 1 IFN receptor [103,104]. Type I IFNs include seven subtypes: IFN- α , IFN- β , IFN- ϵ , IFN- κ , IFN- ω , IFN- δ , and IFN- τ [105]. It has long been known that type 1 IFNs, particularly IFN- α , are intimately related to the etiopathogenesis of SLE. Accordingly, IFN- α -induced gene expression (the so-called “IFN- α signature”) was up-regulated in SLE patients-derived MNCs and in animal models of SLE [81,103,106]. Moreover, IFN- α levels in SLE are remarkably increased due to exacerbated or chronic stimulation of antigen-presenting cells (APCs), such as plasmacytoid dendritic cells (pDCs), by ICs and damage-associated molecular pattern molecules (DAMPs) [104]. Of note, IFN- α inhibits angiogenesis by impairing endothelial cell proliferation and migration through the up-regulation of multiple angiostatic genes, including guanylate-binding protein 1, TRAIL, CXCL10 and CXCL11 [107]. Furthermore, a recent study revealed that IFN- α interferes with endothelial cell growth and tube formation by inducing promyelocytic leukemia protein (PML) to initiate a positive feedforward loop with Stat1/2 and to establish a negative feedback loop with Stat3 [108]. Intriguingly, IFN- α may also dampen endothelial regeneration and vascular repair by reducing the frequency and angiogenic activity of circulating EPCs [5,8,26,34,103].

It has long been known that a reduction in MACs levels in peripheral blood predicts higher occurrence of cardiovascular events [109], while increased circulating MACs/ECFCs are a common feature in cancer patients [110,111]. It was initially reported that the reduced number of MACs in peripheral blood independently predicted major cardiovascular events and could identify patients with increased cardiovascular risk [109]. Accordingly, Hill and colleagues confirmed that MACs were less numerous in subjects affected by high cardiovascular risk factors and displayed significant *in vitro* senescence [112]. Different studies reported an inverse correlation between several cardiovascular risk factors, such as obesity, dyslipidemia, diabetes, smoke, ageing, and increased homocysteine, and MACs/ECFCs frequency and reparative ability [28,30,34,113–117]. In the following paragraph, we describe the impairment of the endogenous mechanisms of vascular repair by MACs and ECFCs in SLE patients.

3. MACs and ECFCs are dysfunctional in SLE patients

Early studies, aiming at assessing whether and how SLE could affect vascular integrity by interfering with circulating EPCs, exploited multiple markers and methodological procedures to isolate EPCs.

3.1. MAC dysfunction in SLE patients

The EPC subtype that has been characterized in these earlier reports is represented by MACs, which were sorted as CD34⁺ cells in combination with additional targets, such as KDR (Kinase Insert Domain Receptor or VEGFR-2) and CD133 [26,34]. As summarized in Table 1, the frequency of CD34⁺ MACs was significantly decreased in peripheral blood of SLE patients as compared to healthy donors in the majority of reports. Nevertheless, two independent studies reported an increase in circulating CD34⁺/CD133⁺/KDR⁺ cells [118] and CD34⁺/CD31⁺/CD133⁺ cells [119], respectively. This discrepancy has been attributed to the heterogeneous selection of SLE patients (different comorbidity

Table 1
Endothelial Progenitor Cells in SLE patients.

Ref.	Nr. of subjects (SLE/HC)	Disease activity State	MAC/ECFC Identification	MAC/ECFC frequency in SLE	Impairment of MAC/ECFC angiogenic activity
[125]	70 SLE and 31 HC	N/A	CD34 ⁺ /KDR ⁺ Dil-Ac-LDL-UEA-lectin	↓ MACs/ECFCs	↓ MACs proliferation; ↓ vascular reactivity in patients with high IFN-α level
[120]	44 SLE and 35 HC	Inactivity	CD34 ⁺ /CD133 ⁺	↓ MACs	↓ migration; ↑ caspase 8 L activity
[121]	15 SLE and 15 HC	Clinical Remission	CD34 ⁺ /KDR ⁺	↓ MACs	↑ Annexin V binding and apoptosis
[33]	135 SLE and 60 HC	Mixed Population	CD34 ⁺ /CD133 ⁺	↓ MACs/ECFCs	↑ apoptosis; ↑ MACs differentiation towards a non-angiogenic phenotype; ↓ VEGF and HGF release; Impairment of endothelial monolayers
[126]	31 SLE and 14 HC	Mixed Population	CD34 ⁺ /KDR ⁺ /CD133 ⁺	Unchanged MAC levels	↓ MAC migration and adhesion; PlGF inversely correlated with MAC frequency
[118]	61 SLE and 20 HC	Mixed Population	CD31 ⁺ /CD34 ⁺ /CD133 ⁺ /CD45 ⁺	↑ MACs	↓ ECFC proliferation and migration
[131]	35 SLE and 35 HC	Active State	CD34 ⁺ /Dil-Ac-LDL/UEA-lectin	Unchanged ECFCs	↑ ECFCs adhesion capacity
[245]	31 SLE and 54 HC	Mixed Population	CD31 ⁺ /Dil-Ac-LDL/UEA-lectin	Unchanged ECFCs	↓ MAC proliferation and migration; ↑ MAC apoptosis
[122]	13 SLE and 13 HC	Inactivity	CD34 ⁺ /KDR ⁺	↓ MACs	IL-1β restored MAC/ECFC proliferation and prevented apoptosis
[127]	11 SLE and 17 HC	Mixed Population	Dil-Ac-LDL/UEA-lectin	↓ MACs/ECFCs	IL-1β ↓ and IL-18 ↑ by INF-α induces MAC/ECFC dysfunction; Caspase-1 inhibition improves MACs/EPCs function
[128]	43 SLE and 37 HC	Mixed Population	Dil-Ac-LDL/UEA-lectin	↓ MACs/ECFCs	N/A
[119]	83 SLE and 85 HC	Mixed Population	CD34 ⁺ /KDR ⁺ /CD133 ⁺ CD34 ⁺ /KDR ⁺	↑ MACs	Osteoprotegerin induced MAC apoptosis through ROS/NADPH oxidase and Nox2/4 ERK/MAPK signaling
[129]	91 SLE and 50 HC	N/A	CD34 ⁺ /KDR ⁺	↓ MACs	IL-10 correlates with MAC/ECFC function in SLE but not in HC by preventing endothelial differentiation
[132]	37 SLE and 21 HC	Mixed Population	Dil-Ac-LDL/UEA-lectin	N/A	↑ apoptosis ↑ IFN-α signature
[130]	18 SLE and 8 HC	Active State	CD34 ⁺ /KDR ⁺	↓ MACs	↑ IFN-α signature
[246]	42 SLE and 63 HC	Active State	CD34 ⁺ /CD133 ⁺	↓ MACs	
[247]	132 SLE and 178 HC	Active State	CD34 ⁺ /CD133 ⁺	↓ MACs	

Table legend: SLE: Systemic Lupus Erythematosus; HC: Healthy Controls; N/A: Not applicable or not specified data; KDR: Kinase Insert Domain Receptor; Dil-Ac-LDL/UEA: Dioctadecyl-Tetramethylindolyl Carbocyanine Perchlorate Labeled Acetylated LDL, Ulex europeus agglutinin; MACs: Myeloid Angiogenic Cells; ECFCs: Endothelial Colony Forming Cells; IFN-α: Interferon-α; VEGF: Vascular Endothelial Growth Factor; HGF: Hepatic Growth Factor; PlGF: placental growth factor; ROS: Reactive Oxygen Species; NADPH: Nicotinamide Adenine Dinucleotide Phosphate; ERK: Extracellular Signal-Regulated Kinase; MAPK: Mitogen Activated Protein Kinase.

Fig. 1. Mechanisms and consequences of MAC/ECFC dysfunction in systemic lupus erythematosus. Systemic Lupus Erythematosus (SLE) is characterized by high IFN-α levels that modulate MAC/ECFC angiogenic activity through the JAK/STAT pathway which down-regulates IL-1β and up-regulates IL-18 signaling. Osteoprotegerin, which is also increased in SLE, binds to syndecan-4, activates the ERK/MAPK phosphorylation cascade and in turn triggers ROS generation by NADPH oxidase. As a result, repression of IL-1β activity, upregulation of IL-18, increased levels of IL10 and down-regulation of proangiogenic factors, such as VEGF and HGF, reduce proliferation, migration and adhesion properties of MACs/ECFCs. Other consequences include apoptosis, impairment of maturation and endothelial monolayer development.

cohorts, therapies and disorder activity) and to the different protocols used for EPC isolation and identification [34]. The reduction in MACs frequency was associated to increased apoptosis due to caspase 8 L activation [120–122]. Likewise, an increased number of apoptotic CD34⁺ cells was reported in the bone marrow of SLE patients [123], whereas apoptosis occurred in bone marrow-derived CD34⁺ in the presence of sera harvested from leukopenic SLE patients [124]. Interestingly, decreased MACs levels were also found in patients with quiescent disease [120] or disease in remission [121]. This evidence

sparked the hypothesis that MACs frequency is chronically reduced throughout this disease, thereby being intimately involved in SLE pathogenesis [26]. Furthermore, the angiogenic activity was severely compromised in SLE-derived MACs. For instance, three independent studies revealed that MACs derived from SLE patients displayed a significant reduction in their CFU ability after 5 and 7 days in culture [120,125,126]. In addition, Moonen and colleagues reported that SLE-derived CFU contained an higher fraction of less-spindled cells, thereby hinting at a change in MACs morphology [120]. Moreover, the release

of pro-angiogenic cues, such as vascular endothelial growth factor (VEGF) and hepatocyte growth factor (HGF), was significantly reduced [33], whereas VEGF-induced proliferation and migration were impaired in SLE-derived MACs [122,126]. In agreement with these observations, the adhesion to an endothelial monolayer, which is an essential step during vasculogenesis, was dampened in SLE-derived MACs [126]. The impairment of MACs function was mostly triggered by IFN- α [34], as suggested by several pieces of evidences. First, IFN- α production was up-regulated in SLE-derived MACs, thereby impairing VEGF and HGF release and reducing their pro-angiogenic activity [33]. Second, IFN- α was able to induce apoptosis [33] or to reduce proliferation [125] in SLE-derived MACs. Third, the angiogenic phenotype was rescued by pre-treating MACs with a monoclonal antibody selectively neutralizing IFN- α [33,125]. Fourth, IFN- α inhibited the pro-angiogenic transcriptional signature promoted by interleukin-1 β (IL-1 β), caused a decrease in VEGF expression, and up-regulated the inflammasome machinery by boosting interleukin 18 (IL-18) signaling in a mixed population of MACs/ECFCs [127,128]. Of note, serum levels of IL-18 were significantly enhanced in SLE patients [128], while exogenous administration of IL-1 β restored proliferation and dampened apoptosis in SLE-derived MACs/ECFCs [127]. In addition to IFN- α , MACs could be affected by osteoprotegerin (OPG), whose levels were increased in the serum of SLE patients and inversely correlated to MACs frequency [129]. Accordingly, OPG ligation via the heparin sulfate proteoglycans, syndecan 2 and syndecan 4, induced MACs apoptosis by promoting reactive oxygen species (ROS) production in a NADPH oxidase-dependent manner [129]. Finally, a recent study disclosed that MAC apoptosis could also be induced by B lymphocyte stimulator (BLyS), a B cell survival factor which undergoes a remarkable increase in serum of SLE patients and has been related to the disorder progression [130]. The mechanisms whereby SLE may affect MACs were summarized in Fig. 1.

3.2. ECFC dysfunction in SLE patients

Less studies were devoted to assessing whether and how SLE affects ECFCs. Early work revealed a significant reduction in EPC numbers when comparing a mixed population of MACs/ECFCs isolated from SLE patients with healthy controls [33]. Furthermore, SLE-derived MACs/ECFCs were less prone to develop endothelial monolayers and to secrete pro-angiogenic cues, such as VEGF and HGF [33]. In agreement with this observation, adhesion, proliferation, migration and tube formation were dramatically reduced in ECFCs isolated from SLE patients [131]. As described above, IFN- α impaired ECFCs-mediated vascular repair by promoting apoptosis, interfering with the pro-angiogenic effect of IL-1 β , and boosting IL-18-induced inflammasome activation [127,128]. Likewise, targeting IFN- α with a neutralizing antibody restored the angiogenic activity in a mixed MACs/ECFCs population [33]. Moreover, interleukin-10 (IL-10), whose levels are increased in SLE, hampered endothelial differentiation and ECFCs-induced neovascularization in Matrigel plugs *in vivo*, thereby supporting the causative role of IFN- α -mediated endothelial dysfunction [132]. An additional source of damage for MACs and ECFCs could be represented by the heightened oxidative stress that has been reported in SLE patients [81,133]. It has indeed been shown that oxidative stress decreased the proliferative potential of both ECFCs [134,135] and MACs [136,137], thereby impairing the endogenous mechanisms of vascular repair.

In the following paragraphs, we speculate about the hypothesis that dysregulation of the intracellular Ca^{2+} toolkit could contribute to impair vascular repair in SLE patients by affecting ECFCs and MACs. We further discuss the possibility to restore the pro-angiogenic activity of circulating EPCs by targeting their Ca^{2+} handling machinery.

4. The role of intracellular Ca^{2+} signaling in ECFCs and MACs

It has long been known that endothelial Ca^{2+} signals play a crucial

role in angiogenesis by controlling endothelial cell adhesion, proliferation, permeability migration, and tube formation [14,138,139]. VEGF elicits distinct endothelial Ca^{2+} signatures depending on the vascular bed [140–147], whereas VEGF-induced intracellular Ca^{2+} oscillations sustain sprouting angiogenesis in zebrafish vasculature [148,149]. Likewise, endothelial Ca^{2+} signals mediate the pro-angiogenic effect of a plethora of growth factors and chemokines, including basic fibroblast growth factor (bFGF) [150], insulin-like growth factor-I (IGF-I) [150], epidermal growth factor (EGF) [151], platelet-derived growth factor (PDGF) [152], stromal derived factor-1 α (SDF-1 α) [76], and angiopoietin-2 (ANG-2) [153]. Multiple Ca^{2+} -dependent decoders, including nuclear factor of activated T-cells (NFAT), Nuclear factor kappa enhancer binding protein (NF- κ B), Ca^{2+} /CaM-dependent protein kinase II (CaMKII), extracellular signal-regulated kinases 1/2 (ERK 1/2), phosphoinositide-3-kinase (PI3K)/Akt, and endothelial NO synthase (eNOS), translate endothelial Ca^{2+} signals into a pro-angiogenic output [14,154]. Similarly, emerging evidence demonstrated that an increase in intracellular Ca^{2+} concentration ($[\text{Ca}^{2+}]_i$), that can adopt distinct waveforms depending on the activating cue, finely regulated the angiogenic activity in human ECFCs and rodent MACs [154,155]. Intriguingly, the Ca^{2+} handling machinery is deregulated in ECFCs/MACs under different pathological conditions, e.g. cancer, primary myelofibrosis, AMI and atherosclerosis, thereby altering their angiogenic activity and contributing to the pathogenesis of the disease [155,156]. The mechanisms whereby SLE may affect ECFCs were summarized in Fig. 1.

4.1. Pro-angiogenic role of the intracellular Ca^{2+} toolkit in human ECFCs

The homing of EPCs to sites of vascular remodeling requires the timely and spatially orchestrated interaction between multiple chemokines, e.g. SDF-1 α and insulin-like growth factor 2 (IGF2), and chemokine receptors, e.g. C-X-C chemokine receptor type 4 (CXCR4) and IGF2 receptor (IGF2R), and downstream signaling pathways [76,157,158]. Ischemic tissues release in the blood stream a plethora of chemokines, that stimulate ECFCs/MACs mobilization and drive them to nascent neovessels. The first demonstration of the role played by intracellular Ca^{2+} in vasculogenesis was provided by Maeng and coworkers, who disclosed that IGF2-induced ECFCs vascularization *in vivo* required an increase in $[\text{Ca}^{2+}]_i$ upon recruitment of phospholipase C β 2 (PLC β 2) [159]. Recently, it was shown that also SDF-1 α was able to elicit intracellular Ca^{2+} signals in circulating ECFCs. The Ca^{2+} response to SDF-1 α consisted in a biphasic elevation in $[\text{Ca}^{2+}]_i$ that was initiated by CXCR4 and comprised an initial Ca^{2+} peak which then decayed to a prolonged plateau phase [160,161]. CXCR4 is a Gi Protein-Coupled Receptor that can engage PLC β 2 through the G $\beta\gamma$ dimer [162]. PLC β 2, in turn, cleaves phosphatidylinositol 4,5-bisphosphate (PIP $_2$), a minor phospholipid present in the inner leaflet of the plasma membrane, into the two intracellular second messengers, inositol-1,4,5-trisphosphate (InsP $_3$) and diacylglycerol (DAG) [162]. InsP $_3$ is the major responsible for Ca^{2+} mobilization from the endoplasmic reticulum (ER), the largest endogenous Ca^{2+} store in vascular endothelial cells [139], as well in circulating ECFCs [155]. InsP $_3$ diffuses towards ER cisternae to gate InsP $_3$ receptors (InsP $_3$ Rs), which represent an ubiquitous family of ionotropic Ca^{2+} -permeable channels that are present in three distinct isoforms (InsP $_3$ R1-3) and mediate ER Ca^{2+} release in response to physiological stimulation [163,164]. Circulating ECFCs express all three InsP $_3$ R isoforms [165], although they lack ryanodine receptors (RyRs) [166], which may amplify InsP $_3$ -induced ER Ca^{2+} release through the mechanism of Ca^{2+} -induced Ca^{2+} release [139]. Pharmacological manipulation confirmed that the rapid Ca^{2+} peak triggered by SDF-1 α was mediated by InsP $_3$ Rs [160], while DAG could play no role in the Ca^{2+} signal as circulating ECFCs do not express the DAG-sensitive Transient Receptor Potential (TRP) Canonical (TRPC) 3, 6 and 7 (TRPC3, TRPC6, TRPC7) [167,168].

The plateau phase that followed SDF-1 α -induced intracellular Ca^{2+}

peak was maintained by extracellular Ca^{2+} influx through the mechanism of store-operated Ca^{2+} entry (SOCE) [160]. Briefly, InsP_3 -induced Ca^{2+} release leads to a robust depletion in ER Ca^{2+} concentration ($[\text{Ca}^{2+}]_{\text{ER}}$) that must be replenished by the influx of extracellular Ca^{2+} . It has been demonstrated that any fall in ER Ca^{2+} concentration ($[\text{Ca}^{2+}]_{\text{ER}}$) is detected by Stromal Interaction Molecule (STIM) proteins, which function as ER Ca^{2+} sensors and are activated following reduction in ER Ca^{2+} levels [169]. Thereafter, STIM proteins, which exist in two distinct isoforms (STIM1 and STIM2), oligomerize and relocate from perinuclear to peripheral ER cisternae, which are located in close proximity (10–20 nm) from the plasma membrane. A these ER-plasma membrane junctions, also known as *puncta*, STIM proteins tether and gate the hexameric Ca^{2+} -permeable Orai channels, thereby promoting SOCE activation and ER Ca^{2+} refilling [169]. In addition, multiple Ca^{2+} -dependent decoders are positioned nearby the cytosolic mouth of Orai hexamers and are invested by the sub-membranal Ca^{2+} microdomain arising upon Orai opening. Therefore, Orai-mediated SOCE regulates additional cellular processes, including proliferation, migration, and gene expression [170,171]. Three Orai isoforms (Orai1–3) are known and able to interact with STIM1–2 proteins. Based on their different ER Ca^{2+} sensitivity and Orai gating ability, it has been proposed that STIM2 maintains constitutive ER Ca^{2+} entry and is engaged by moderate ER Ca^{2+} depletion, while STIM1 is activated following extensive ER Ca^{2+} mobilization [172]. Circulating ECFCs express Orai1, Orai2 and Orai3 [167], although agonist-induced SOCE is mediated by the dynamic interplay between STIM1 and Orai1 [173,174]. However, STIM1 and Orai1 must interact with TRPC1 to mediate SOCE, as also shown in several types of vascular endothelial cells [175–177], in platelets [178], megakaryocytes [179], and salivary gland cells [180]. The Ca^{2+} response to SDF-1 α in ECFCs was dramatically curtailed by pharmacological blockade of SOCE and adopted the single transient waveform recorded when extracellular Ca^{2+} was omitted by the bathing solution [160]. Of note, ECFCs migration *in vitro* and neovessel formation in Matrigel plugs *in vivo* were abrogated by preventing the ensuing Ca^{2+} response to SDF-1 α . It was shown that InsP_3 -induced ER Ca^{2+} release and SOCE engaged the ERK and PI3K/Akt phosphorylation cascades to promote SDF-1 α -induced motility [160]. Interestingly, ECFC migration was also sustained by ATP-dependent K^+ (K_{ATP}) channels, which are likely to increase the driving-force sustaining Ca^{2+} entry by hyperpolarizing the membrane potential [181]. In agreement with this hypothesis, pharmacological stimulation of K_{ATP} channels stimulated ECFCs to migrate by recruiting the pro-angiogenic Ca^{2+} -dependent decoders, eNOS and CaMKII [181].

Once reached the target site, ECFCs proliferate and, then, may physically incorporate within the nascent neovessel [158]. VEGF drives proliferation and tubulogenic activity in circulating ECFCs by binding to the cell surface receptor, VEGFR2 [30,162,182]. VEGF induced intracellular Ca^{2+} oscillations by triggering repetitive cycles of InsP_3 -dependent ER Ca^{2+} release, each followed by Ca^{2+} sequestration into ER lumen by Sarco-Endoplasmic Reticulum Ca^{2+} -ATPase 2 B (SERCA2B), thereby reloading the store to generate the next Ca^{2+} transient [154,165]. SOCE was required to support the spiking Ca^{2+} signal and to support the nuclear translocation of the Ca^{2+} -sensitive transcription factor, NF- κB [165]. Consistently, pharmacological blockade of intracellular Ca^{2+} oscillations and NF- κB activation prevented VEGF-induced proliferation and tube formation in circulating ECFCs [165]. The intimate relationship between Ca^{2+} signaling and proliferation is corroborated by the notion that the higher proliferative potential is associated to more robust VEGF-induced intracellular Ca^{2+} oscillations in umbilical cord derived-ECFCs [168,183]. Similar to migration, K_{ATP} channels support ECFC proliferation by favoring the Ca^{2+} -dependent engagement of eNOS and CaMKII [181].

In addition to growth factors and chemokines, ischemic tissues may liberate significant amounts of arachidonic acid (AA). For instance, brain damage and AMI may induce a remarkable increase in local AA levels, that may raise by 10-to-13 fold from a resting concentration of

$\approx 0.5\text{--}20\text{ }\mu\text{M}$ [162,184,185]. Likewise, physical exercise, that has long been known to increase ECFCs frequency in peripheral blood [186], may elevate AA concentration in skeletal muscle up to $\approx 500\text{ }\mu\text{M}$ [187]. It has long been known that AA stimulates endothelial signaling through an elevation in $[\text{Ca}^{2+}]_i$ [188–190]. For instance, AA elicited endothelial Ca^{2+} signals during the early phases of tubulogenesis in breast cancer-derived endothelial cells [188] and mediated the Ca^{2+} response to bFGF in healthy endothelial cells [14]. A recent investigation demonstrated that AA was able to stimulate ECFCs proliferation in a Ca^{2+} -dependent manner [166]. AA evoked endogenous Ca^{2+} release by mobilizing both the ER and the endolysosomal (EL) Ca^{2+} stores. The acidic vesicles of the EL system are emerging as a crucial signaling hub for Ca^{2+} -mediated events across the phylogenetic tree [191–193] and mediate multiple endothelial functions, including angiogenesis [194], secretion of von Willebrand factor [195] and NO release [196,197]. AA was found to stimulate ER Ca^{2+} release through InsP_3Rs and EL Ca^{2+} mobilization through two-pore channel 1 (TPC1) in ECFCs [166], although the underlying gating mechanisms remain unclear [162]. Furthermore, AA induced extracellular Ca^{2+} entry through TRP Vanilloid 4 (TRPV4), which represents an ubiquitous Ca^{2+} entry gated by AA in vascular endothelium [190,198]. AA evoked a long-lasting Ca^{2+} signal that recruited eNOS to stimulate ECFC proliferation [166], thereby highlighting an alternative strategy to promote vascular repair through local AA delivery [162].

4.2. Pro-angiogenic role of the intracellular Ca^{2+} toolkit in rodent MACs

The pro-angiogenic role of Ca^{2+} signaling in MACs has been investigated in rat and mouse bone marrow-derived cells. Early work revealed that SOCE was mediated by the physical interaction between STIM1 and TRPC1 in rat MACs, while the contribution of Orai1 was not evaluated [199]. Genetic silencing of either STIM1 or TRPC1 hampered proliferation, migration, and tubulogenesis [199], thereby confirming the pro-angiogenic role of SOCE also in rat MACs. Inhibition of TRPC1 expression induced the cell cycle to arrest in the G1 phase due to the up-regulation of 9 genes, including Ak1, Brca2, Camk2b, p21, Ddit3, Inha, Slfn1, Mdm2, Prm1, and the down-regulation of other 4 genes, such as Pmp22, Ppp2r3a, Mki67 and Bcl2 [199]. This study suggested that Slfn1 was the main responsible for the anti-proliferative effect of TRPC1 knockdown. A Slfn-1 blocking peptide rescued the effect of TRPC1 silencing on cell cycle distribution and proliferation, thereby suggesting that TRPC1 blocks cell growth by repressing Slfn1 expression in rat MACs [199]. A follow-up investigation confirmed that Slfn1 upregulation inhibited proliferation and tube formation by increasing the expression of Cyclin D1 [200]. These findings were in agreement with previous reports demonstrating that Slfn1 controls cell cycle by modulating Cyclin D1 levels [201,202]. Furthermore, HGF-induced rat MACs proliferation was impaired by pharmacological blockade of SOCE and genetic silencing of STIM1 [203]. SOCE controlled proliferation and bidimensional tube formation also in murine MACs [204]. Indeed, rat MACs expressed STIM1, TRPC1 and Orai1, whereas pharmacological blockade of SOCE and genetic silencing of STIM1 inhibited their angiogenic behavior by impairing eNOS activation. Furthermore, VEGF induced repetitive oscillations in $[\text{Ca}^{2+}]_i$ that resembled those evoked in human ECFCs [204]. In line with these observations, VEGF stimulated human MACs to proliferate by promoting the nuclear translocation of the Ca^{2+} -sensitive transcription factor, NFAT2, which led to an increase in eNOS expression and NO release [205]. These preliminary findings confirm that the intracellular Ca^{2+} toolkit also modulates the reparative phenotype of MACs, although it remains to be elucidated whether SOCE drives angiogenesis in humans.

5. The rationale for dysregulation of the intracellular Ca^{2+} machinery in SLE-derived ECFCs and MACs

Several pieces of evidence suggest that the Ca^{2+} handling

machinery could be remodeled in SLE-derived ECFCs/MACs. First, it has long been known that the endothelial Ca^{2+} toolkit is affected by multiple cardiovascular risk factors, including atherosclerosis [206], oxidative stress [207], diabetes [208–211], obesity [212], and aging [213]. For instance, STIM1 was down-regulated by type 1 diabetes in mouse coronary artery endothelial cells [210], while hyperglycemia caused an increase in TRPC1 expression and SOCE amplitude in bovine aortic endothelial cells [208]. Furthermore, high glucose enhanced SOCE by inducing the up-regulation of STIM1 and Orai1 proteins in human aortic endothelial cells [209]. Conversely, hypercholesterolemia dampened SOCE and turned the biphasic Ca^{2+} response to carbachol into an oscillating signal in vascular endothelium of rat thoracic aorta [206]. Likewise, oxidative stress imposed by the prolonged application of hydrogen peroxide (H_2O_2) reduced SOCE, thereby resulting in the following depletion of the ER Ca^{2+} store [214]. In addition, H_2O_2 promoted endothelial cell apoptosis by inducing cytosolic Ca^{2+} overload through the ROS-sensitive TRP Melastatin 2 (TRPM2) channel [215]. Of note, the production of mitochondrial ROS could be boosted during endothelial cell aging as a consequence of enhanced InsP_3 -dependent ER-to-mitochondria Ca^{2+} transfer, thereby contributing to senescence-induced endothelial dysfunction [213]. Conversely, local InsP_3 -dependent endothelial Ca^{2+} pulsars arising at the myo-endothelial junctions to control vascular tone via endothelium-dependent hyperpolarization (EDH) were down-regulated in mesenteric arteries of aging mice [216].

Second, the Ca^{2+} machinery could also be rewired by the pathological background of the donor also in ECFCs and MACs. For instance, SOCE was up-regulated in renal cellular carcinoma (RCC)-derived ECFCs due to the over-expression of STIM1, Orai1 and TRPC1 [173]. STIM1 was up-regulated also in breast cancer (BC)-derived ECFCs, although there was no difference in SOCE amplitude [217]. Interestingly, STIM1, Orai1 and TRPC1 were not up-regulated in infantile hemangioma (IH)-derived ECFCs. However, SOCE was constitutively activated due to the partial depletion of the ER Ca^{2+} store [218]. Of note, constitutive SOCE increased basal levels of NO release in IH-ECFCs, which enhanced their proliferation rate as compared to their healthy counterparts and could be involved in the aberrant vascularization that features hemangioma lesions [218]. This hypothesis is supported by the recent finding that propranolol, a β -adrenergic receptor antagonist that is widely employed to treat IH, reduced ECFC frequency in IH patients by inhibiting constitutive SOCE [219].

In addition, cardiovascular risk factors could affect the Ca^{2+} handling machinery in endothelial precursors. VEGF-induced InsP_3 -dependent Ca^{2+} release and SOCE were remarkably enhanced in ECFCs harvested from AMI patients at day 0, while these processes were not significantly different from healthy controls at days 7 and 180 [220]. The enhanced Ca^{2+} response to VEGF could be involved in the transient increase in ECFC frequency reported in peripheral blood of AMI patients [221]. Conversely, SOCE was dampened in bone marrow-derived MACs isolated from atherosclerotic mice. The partial inhibition of SOCE was associated to the down-regulation of STIM1, Orai1 and TRPC1 and led to the impairment of proliferation and migration by tempering VEGF-induced intracellular Ca^{2+} oscillations and NO release [204]. This observation is quite remarkable as it suggests that SOCE dysregulation could be involved in the reduced regenerative potential of MACs (and, possibly, ECFCs) also in SLE. On the other hand, oxidative stress could activate SOCE to promote apoptosis in rat MACs. A recent investigation revealed that H_2O_2 induced endogenous Ca^{2+} release followed by SOCE activation [222], thereby promoting ROS production, mitochondrial dysfunction and apoptosis. This finding is in agreement with the notion that H_2O_2 is able to directly stimulate InsP_3 -dependent Ca^{2+} release in vascular endothelial cells [223]. The genetic silencing of STIM1 and pharmacological blockade of SOCE prevented the detrimental effects of H_2O_2 on MACs' fate [222].

Third, remodeling of the Ca^{2+} toolkit has been reported in SLE-derived T and B cells [224,225]. For instance, B cell receptor (BCR)

stimulation induced larger InsP_3 -dependent ER Ca^{2+} release in SLE-derived B cells [226]. However, the enhanced Ca^{2+} response was due to defective $\text{Fc}\gamma\text{RIIb}$ receptor-dependent inhibition of InsP_3 degradation by the phosphatase SH2 domain-containing inositol 5'-phosphatase (SHIP) [227]. Likewise, an increase in intracellular Ca^{2+} mobilization has been observed in CD4^+ and CD8^+ T cells following anti-CD3 stimulation, although there was no difference in the releasable ER Ca^{2+} pool between healthy and SLE-derived T cells. Of note, the long-lasting plateau phase that follows the initial Ca^{2+} peak and is due to Orai1-mediated extracellular Ca^{2+} entry was dramatically down-regulated in the activated T cells [228]. This early investigation suggested that SOCE could be decreased by SLE in human subjects. The relationship between SLE and SOCE has been further highlighted by two recent investigations. It has first been disclosed that SOCE protects Jurkat T cells from IFN- α -induced apoptosis and inhibits IFN- α -induced transcriptional response by recruiting NF- κB [229]. Conversely, overexpression of the dominant negative Orai1-E106A to inhibit SOCE, sensitized Jurkat T cells to IFN- α -induced apoptosis [229]. Subsequently, STIM1 was shown to retain the signaling adaptor Stimulator of interferon genes (STING) within the ER membrane, thereby preventing its relocation to the ER-Golgi intermediate compartment. Herein, STING could recruit TANK-binding kinase 1 (TBK1) and interferon regulatory factor 3 (IRF3) to initiate type I IFN responses [230]. As a consequence, STIM1 deletion resulted in enhanced anti-inflammatory signaling induced by type I IFN both *in vitro* and *in vivo* [230].

Based on these evidences, it is feasible to speculate that, if SOCE is chronically inhibited in SLE-derived EPCs (as demonstrated in atherosclerotic MACs, see above), this would lead to a significant reduction in their reparative ability and to an increase in IFN- α -induced apoptosis

Fig. 2. A hypothesis on the double edged (causative and therapeutic) role of intracellular Ca^{2+} signaling in systemic lupus erythematosus. SOCE could be downregulated by chronically elevated IFN- α levels, thereby reducing the pro-angiogenic activity in MACs and ECFCs. Nevertheless, enhanced oxidative stress, in the form of ROS, could activate SOCE, thereby increasing the oxidative damage. The intracellular Ca^{2+} machinery could, however, be properly targeted to restore (at least partially) the angiogenic activity in ECFCs, which represent the most suitable EPC subtype for regenerative purposes. Light-induced excitation of TRPV1 and genetic manipulation of TRPC3 levels may be considered as plausible strategies in restoring ECFCs' angiogenic activity in SLE. For instance, the combined use of optical excitation and organic polymers technology could stimulate circulating ECFCs in SLE patients, thereby activating TRPV1. The following influx of Ca^{2+} could, in turn, recruit Ca^{2+} -sensitive decoders, such as NF- κB , to stimulate ECFC proliferation and tube formation. Alternately, gene manipulation could be combined with autologous ECFC therapy. It has been suggested that ECFCs could be engineered with a TRPC3-expressing plasmid before being re injected into the patients. As TRPC3-expressing ECFCs readily respond to VEGF, this strategy could improve the vasoreparative potential of SLE-derived ECFCs.

(Fig. 2). At the same time, the residual SOCE activity could be diverted by the enhanced oxidative stress from downstream pro-angiogenic pathways and redirected towards pro-apoptotic effectors, thereby boosting cell death in both MACs and ECFCs (Fig. 2). Therefore, SOCE down-regulation could underlie the impairment of the endogenous mechanisms of vascular repair observed in SLE patients.

6. Targeting the intracellular Ca^{2+} toolkit to restore the angiogenic activity in ECFCs

While rewiring of their Ca^{2+} handling machinery in SLE remains to be demonstrated, several investigations suggested how to target the intracellular Ca^{2+} toolkit to stimulate proliferation and tube formation in circulating ECFCs [162,183]. This strategy has been supported by the evidence that several members of the TRP super-family of Ca^{2+} -permeable channels may be engaged to stimulate angiogenesis and restore local blood flow in several animal models of ischemic disorders. For instance, intraperitoneal injection of evodiamine, a TRP Vanilloid 1 (TRPV1) agonist, induced an increase in capillary density in Matrigel plugs *in vivo* by recruiting eNOS and boosting NO release [231]. Likewise, stimulating TRPV4 with 4 α -phorbol 12,13-didecanoate (4 α -PDD) promoted brain angiogenesis and rescued neurological damage in a rat model of transient brain ischemia [232]. Moreover, inducing TRPC5-mediated extracellular Ca^{2+} entry with riluzole induced neo-vascularization and restored local blood flow in a murine model of hindlimb ischemia [233]. The same effect was obtained upon local administration of an adeno-associated virus construct encoding for TRPC5 [233]. Finally, TRPC3 contributes to trigger the intracellular Ca^{2+} oscillations that drive *in vitro* tube formation in the Ea.hy926 endothelial cell line [234]. These evidences support the view that endothelial Ca^{2+} signaling may be exploited to promote vascular repair by stimulating angiogenesis.

6.1. What about vasculogenesis, then?

ECFCs may stimulate vascular regeneration by paracrine stimulation of local endothelial cells and by physically engrafting within neovessels. Therefore, it has been suggested that ECFCs stimulation represents the most effective strategy to promote endothelial repair [22,23,53,55]. An early series of studies suggested that the reparative phenotype of circulating ECFCs could be rescued by TRPC3 over-expression [183]. As mentioned above, TRPC3 is a DAG-sensitive Ca^{2+} -permeable channel that supports VEGF-induced Ca^{2+} entry and angiogenesis in multiple endothelial cell types, including human umbilical vein endothelial cells [235] and human microvascular endothelial cells (HMVECs) [236]. TRPC3 is absent in circulating ECFCs, while it is heavily expressed in umbilical cord blood-derived ECFCs [168]. In these cells, VEGF-induced intracellular Ca^{2+} oscillations were triggered by TRPC3-mediated Ca^{2+} entry and thereafter shaped by the rhythmic interaction between InsP_3 -dependent Ca^{2+} release and SOCE [168]. The addition of TRPC3 to the Ca^{2+} signaling machinery recruited by TRPC3 remarkably increased the frequency of VEGF-induced repetitive Ca^{2+} spikes, thereby leading to the hypothesis that the higher proliferation rate observed in umbilical cord blood-derived ECFCs was due to TRPC3 activation [24,40]. It has, therefore, been suggested that genetic manipulation of endogenous ECFCs by transfecting a TRPC3-encoding construct before reinjection into the patient could rejuvenate their reparative phenotype (Fig. 2) [162,183]. Of note, ECFCs may be efficiently engineered to express pro-angiogenic transgenes by using multiple viral vectors, including adenoviruses, lentiviruses, and retroviruses [55,237,238]. In addition, the lentivirus transduction efficiency is remarkably higher in circulating ECFCs as compared to bone marrow-derived MACs [239], a feature which renders ECFCs particularly suitable for genetic manipulation [55].

An additional evidence in favor of the hypothesis that the intracellular Ca^{2+} toolkit is a suitable target to stimulate endogenous

ECFCs has been provided by Balbi and coworkers [240]. These authors demonstrated that injection of the secretome collected from umbilical cord blood-derived mesenchymal stem cells maintained under hypoxic conditions was able to restore the coronary vascular network and rescue myocardial function in a murine model of AMI. The hypoxic secretome was shown to induce *in vitro* tube formation by eliciting repetitive Ca^{2+} transients in human ECFCs; this signal strongly resembled the oscillatory response to VEGF, although the underlying signaling machinery was not evaluated [240,241]. This investigation provides the proof of concept that it is possible to exploit the Ca^{2+} toolkit to reactive the endogenous mechanisms of vascular repair by properly stimulating circulating ECFCs (and possibly MACs). Identifying which component of the hypoxic secretome, or which cocktail of components, is more effective at inducing the intracellular Ca^{2+} oscillations in ECFCs could highlight a novel approach to treat endothelial dysfunction-related diseases.

Finally, a recent investigation unveiled an additional mechanism to stimulate proliferation and *in vitro* tube formation by eliciting extracellular Ca^{2+} entry in circulating ECFCs. TRPV1 is a polymodal Ca^{2+} -permeable channel that is sensitive to multiple chemical and physical stimuli, including the dietary agonist capsaicin, extracellular protons, ROS and temperature increases ($> 42^\circ\text{C}$) [242]. Early work demonstrated that TRPV1 was expressed in umbilical cord blood-derived ECFCs, thereby promoting angiogenesis by mediating cellular intake of anandamide [243]. Recently, Lodola and coworkers demonstrated that optical excitation of ECFCs with visible light (520 nm) induced TRPV1-dependent depolarization in circulating ECFCs cultured on the photosensitive conjugated polymer Poly(3-hexyl-thiophene) (P3HT) [244]. Optical excitation indeed resulted in the local production of ROS and increase in temperature at the cleft between the polymer and cell surface, thereby generating a combination of signals converging on TRPV1 activation [244]. Of note, long-term optical stimulation increased the proliferation rate and promoted *in vitro* tube formation only when ECFCs were plated on P3HT. In addition, the pro-angiogenic response to photostimulation was abolished by pharmacological blockade of TRPV1 and by buffering intracellular Ca^{2+} with 1,2-bis(*o*-aminophenoxy)ethane-*N,N,N',N'*-tetraacetic acid (BAPTA) [244]. Therefore, optochemical stimulation of TRPV1-dependent extracellular Ca^{2+} signals could provide a promising strategy to stimulate vascular repair and rescue endothelial dysfunction in SLE patients (Fig. 2).

7. Conclusion

Preventing premature cardiovascular disease remains a compelling therapeutic need for the management of SLE patients. Growing evidence hinted at the impairment of the homeostatic vascular repair mechanisms as a crucial step in the pathogenic cascade leading to endothelial dysfunction in this life-threatening disease. Most of the attention has hitherto been paid to MACs, although ECFCs represent the only EPC subtype which is intimately able to engraft within the endothelial monolayer. It is, therefore, mandatory to better understand how ECFCs' reparative phenotype is affected by SLE and to extend the analysis of both MACs and ECFCs to larger cohorts of patients. As the disease is rarer as compared to, e.g. AMI and peripheral artery disease, evaluation of EPC frequency and bioactivity in larger populations of SLE patients represents a challenging task. At the same time, understanding how SLE affects ECFCs is a prerequisite to design more effective strategies for successful treatment of the patients. Herein, we discussed about the hypothesis that SOCE, the most important extracellular Ca^{2+} entry pathway in EPCs, could be at the forefront of EPC impairment. On the one hand, SOCE could be down-regulated by type I IFN, thereby decreasing the pro-angiogenic response in ECFCs and MACs. This hypothesis is also supported by the notion that SOCE is compromised in atherosclerotic MACs. On the other hand, SOCE could be recruited by oxidative stress to exacerbate ECFCs/MACs' dysfunction through an aberrant increase in $[\text{Ca}^{2+}]_i$. While it is not advisable to

inhibit SOCE in SLE, as the residual pro-angiogenic activity in ECFCs and MACs would definitively be abolished, blockade of IFN- α signaling with monoclonal antibodies is predicted to rescue EPC dysfunction, possibly by relieving SOCE down-regulation. It should, however, be pointed out that pharmacological treatments, such as statins, that were shown to increase EPC frequency and activity in cardiovascular disorders did not conclusively prove to be effective also in SLE [8,34,116]. Manipulation of the signaling pathways that drive EPC to the sites of vascular damage, thereby promoting proliferation and incorporation within (ECFCs) or around (ECFCs and MACs) nascent neovessels, provides an alternative approach to treat any disease featured by insufficient vascularization or by endothelial dysfunction [24,26,34,55,135,162]. Preliminary investigations described how to use the endothelial Ca^{2+} toolkit to stimulate ECFCs to undergo proliferation and *in vitro* angiogenesis. These evidences remain to be confirmed *in vivo*, however, they indicate that an alternative pathway to restore ECFCs' reparative phenotype is now open. Future work is necessary to assess whether their Ca^{2+} handling machinery is altered in SLE and how to target intracellular Ca^{2+} signaling to restore the endogenous mechanisms of repair in SLE patients.

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Declaration of competing interest

None.

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