Peripheral Blood Stem Cells of Patients with Systemic Lupus Erythematosus Show Altered Differentiation into Macrophages

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Abstract: Patients with systemic lupus erythematosus (SLE) often display clearance deficiencies for various targets like apoptotic cells. Most of the *in vitro* phagocytosis assays have been performed with leukocyte preparations from patients with SLE and may, therefore, be influenced by immunosuppressive drugs. We isolated circulating hematopoietic stem cells from patients with SLE to generate adherent cells *in vitro* and to evaluate their phenotype in the absence of any therapeutic influence. CD34+/CD45+ stem cells were sorted with a cell sorter out from peripheral blood and cultured in methylcellulose-based medium. Differentiation into adherent cells was induced by culture of colony forming cells in medium containing granulocyte-macrophage colony-stimulating factor (GM-CSF). The cell cycle and cell death of CD34+/CD45+ stem cell-derived cells were not altered in patients with SLE. However, the differentiated cells showed disturbed adhesion and altered morphology in patients with SLE. Adherent cells derived from patients with SLE failed to express CD11, CD14, CD45, and CD16. Furthermore, the phagocytosis of IgG coated beads by stem cell-derived adherent cells was impaired in the case of SLE. We suggest that the impaired clearance of apoptotic cells seen in patients with SLE may involve intrinsic defects in the differentiation of myeloid progenitors.

Keywords: Stem cells, macrophages, SLE, phagocytosis, differentiation.

INTRODUCTION

Systemic lupus erythematosus (SLE) is a multisystem disease characterized by the production of autoantibodies (AAb) targeting nuclear structures and by immune mediated tissue damage. Many aspects of the etiopathogenesis of SLE still remain elusive. Concordance studies in twins indicate that genetics contributes to the pathogenesis of SLE and genomic wide association studies revealed that multiple genes are involved [1]. Most of these genes play important roles in the initiation, execution, and regulation of immune responses. Nevertheless, besides the rare complete deficiency of C1q [2] and C4 [3], none of these genetic alterations alone is sufficient to cause human SLE.

In patients with SLE, apoptotic cells are often not properly cleared by phagocytes [4]. This can be due to an accelerated apoptosis [5], to defects in opsonizing agents [6], and to defects in phagocytozing cells like macrophages [7]. Consequently, autoantigens leak out of the dying cells and are subsequently presented by follicular dendritic cells to autoreactive B cells in secondary lymphoid tissues [8]. This alteration challenges the peripheral T and B cell tolerance [9]. Autoreactive B cell activation and production of antinuclear AAb are markers for the insidious beginning of the disease [10]. The persistence of such autoimmune events over years causes widespread inflammatory responses against various organs [11].

To get further insights whether intrinsic defects of phagocytes contribute to the pathogenesis of SLE, we examined in the current study the phenotype and phagocytosis capabilities of adherent cells differentiated out of circulating hematopoietic stem cells (SCs) of patients with SLE. In humans, a small number of hematopoietic SCs are present in bone marrow. Some of them can also be found in the peripheral blood. When cultured in a suitable semi-solid matrix, these hematopoietic progenitors proliferate and differentiate resulting in the generation of discrete cell clusters or colonies with the potential to generate different hematopoietic descendants under adequate microenviroments [12]. This allows the evaluation of *in vitro* generated phagocytes which have not been in contact with immune suppressive drugs before. The current work may contribute for the further understanding how impaired clearance of apoptotic cells in patients with SLE comes off. We describe for the first time that peripheral blood derived stem cells of patients with SLE show a reduced capability to differentiate into adherent macrophages.

PATIENTS, MATERIALS AND METHODOLOGY

Stem cells were isolated from 9 patients that visited our outpatient clinic and met at least four of the classification criteria of the American College of Rheumatology for sys-

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temic lupus erythematosus [13]. Sex and age matched healthy volunteers served as controls. Both samples were taken and processed simultaneously. A written informed consent was obtained from all blood donors and the study received the final approval from the ethics committee of the University Hospital Erlangen. Heparinized venous blood was collected by venipuncture.

Cell Separation and Stem Cell Sorting

Peripheral blood mononuclear cells (PBMC) were isolated from venous heparinised blood by Ficoll density gradient centrifugation (Lymphoflot; Biotest, Dreieich, Germany). Depletion of platelets was achieved by density gradient centrifugation through a cushion of fetal calf serum (FCS, Gibco, Invitrogen). Circulating stem cells were isolated from PBMC by cell sorting after labelling with fluorescein isothiocyanate (FITC) anti-CD45 antibody, clone HI30, and phycoerythrin (PE) anti-CD34 antibody, clone 581 (both from BD Pharmingen, Erembodegem, Belgium). Stem cells were sorted out in five of the nine patients (30 cells/well and up to 9 wells per donor) directly into 96 well plates previously filled with complete methylcellulose-based medium (MethoCult, StemCell Technologies, Grenoble, France) with a MoFlo cell sorter (Dako Cytomation, Hamburg, Germany). Stem cells were cultured for 14 days at 37°C in a humidified atmosphere containing 5.5% CO_2 in Methocult. Afterwards the cultures were monitored for presence of colony forming units (CFU), harvested, and counted for further analyzes.

Cell Cycle and Cell Death Analyzes

In order to analyze the cell cycle of growing stem cells and for the detection of apoptosis we have measured the nuclear DNA content by propidium iodide (PI) staining as described elsewhere [14]. Briefly, $1x10^5$ harvested colony forming cells (CFC) were incubated in a solution containing 0.1% sodium citrate, 0.1% Triton-X100 and 10 μg/ml PI for 24 hours in the dark at 4°C. Finally, the samples were measured by flow cytometry (EPICS, Beckman Coulter, Cambridge, MA) and the PI-fluorescence of the nuclei was monitored in FL4 channel. The specific peaks of the fluorescence represent the nuclear DNA content of CFC in the different phases of the cell cycle. Furthermore, sub-G1 fluorescence can be considered as DNA degradation, typical for an ongoing apoptotic process.

Differentiation of Stem Cells into Adherent Cells and Measurement of Adhesion

Harvested CFC were transferred into differentiation medium containing RPMI 1640 medium supplemented with 10% FCS, 1% L-glutamine (Gibco Invitrogen), 1% N-2-hydroxyethylpiperazine-N9-2-ethanesulfonic acid (HEPES; Merck, Darmstadt, Germany), 1% penicillin-streptomycin (Gibco Invitrogen), and 10 ng/ml of granulocyte-macrophage colony-stimulating factor (GM-CSF; Behringwerke, Marburg, Germany). Cells were seeded in 96-well plates at $1x10^5$ per well (up to 12 wells per sample) and cultured for 7 additional days. On day 3 and 5 of cultivation, fresh medium was added to the cultures. At day 7, the cultures were washed with warm (37°C) phosphate buffer saline to remove all non adherent cells. These cells were counted and stained as below for analysis in flow cytometer. The morphology of the remaining adherent cells was examined using light microscopy. Afterwards, adherent cells were fixed with 1% Glutaraldehyde and stained with crystal violet (0.05% in 20% Methanol). Finally, the plates were washed by immersion in water. The adherence was quantified with a spectrophotometer (λ=595 nm), measuring the absorbance of with sodium dodecyl sulphate (1%) solubilized crystal violet stained cells as previously described by Gillies and collaborators [15].

Immune Phenotyping

CFCs, CFCs after differentiation, PBMCs, and monocyte-derived macrophages (MoMa) were stained for 30 min with saturating amounts of directly labeled monoclonal antibodies (mAbs). After washing and fixation with 1% paraformaldehyde, at least 20000 cells in the viable cell gate were analyzed by flow cytometry. The following PE or FITC conjugated mAbs were used: anti-CD11b, anti-CD14, anti-CD16, anti-CD34, anti-CD44, anti-CD45, anti-CD86, and anti-HLA-DR (all from BD Pharmingen, Heidelberg, Germany). Unlabeled cells were used as control for autofluorescence and the respective isotype antibodies to determine background fluorescence.

Phagocytosis of IgG Coated Beads

Fluoresbrite® YG beads (1 μm, Polysciences, Warrington, USA) were coated with 10μg/ml human immunoglobulins (Pentaglobin®; Biotest, Vienna, Austria) by passive adsorption for 2 hours in 0.1 M carbonate buffer. Adherent cells of healthy donors and patients with SLE were fed with $2x10^6$ particles/ml culture medium and incubated 1 hour at 37°C. Phagocytosis was evaluated by microscopy.

Statistical Analysis

For the comparison of the adhesion measurements the Student t Test was employed. In all other cases, patients and controls were analyzed in pairs belonging to the day of the experiment.

RESULTS

The Frequency of CD34+/CD45+ Stem Cells in Peripheral Blood and its Growth *in Vitro* are not Altered in Patients with SLE

The frequency of CD34+/CD45+ stem cells in peripheral blood of patients with SLE (0.06 ± 0.07 %) was similar to that of the normal donors (0.05 ± 0.04 %). Sorted circulating human CFC from healthy donors and patients with SLE were cultured in methylcellulose-based complete medium for 2 weeks. The appearance of CFU developed out of 30 seeded stem cells were similar between patient and control wells (Fig. **1A**). Cell cycle analysis of the CFU by flow cytometry demonstrated normal proliferating cells in both healthy donors and patients with SLE. Furthermore, the amount of dead cells was similar, as detected by the subG1 DNA content of the cells (Fig. **1B** and **C**).

Differentiated Cells Derived from CFC Show Disturbed Adhesion and Morphology in Patients with SLE

CFCs were transferred into medium containing GM-CSF and FCS for one additional week to differentiate into adherent cells. The amount of the latter was quantified by the crystal violet assay. The differentiation of CFCs into adherent

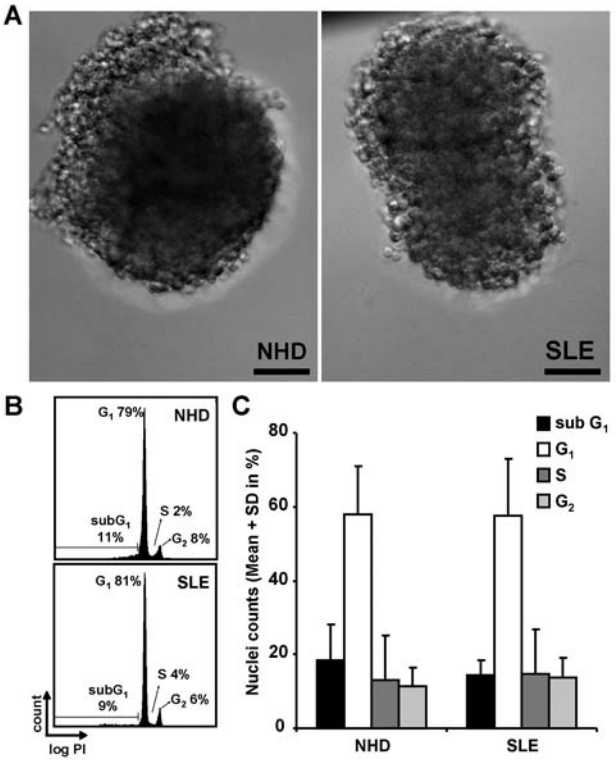


Fig. (1). Cell cycle and cell death of human CFC (CD34+/CD45+ stem cells) isolated from peripheral blood. The appearance of CFU derived from CD34+/CD45+ cells sorted from peripheral blood and cultured in methylcellulose-based complete medium for 2 weeks is shown in (**A**). Cell cycle and cell death analyzes of the CFU after 2 weeks of culture by flow cytometry demonstrate normal proliferating cells in both healthy donors and patients with SLE. The amount of nuclei with subG1 DNA content, a marker of cell death, was also similar in NHD and patients with SLE (C; n=5). Representative histograms of the measurements by flow cytometry are displayed in (**B**). The bar represents 100 μm.

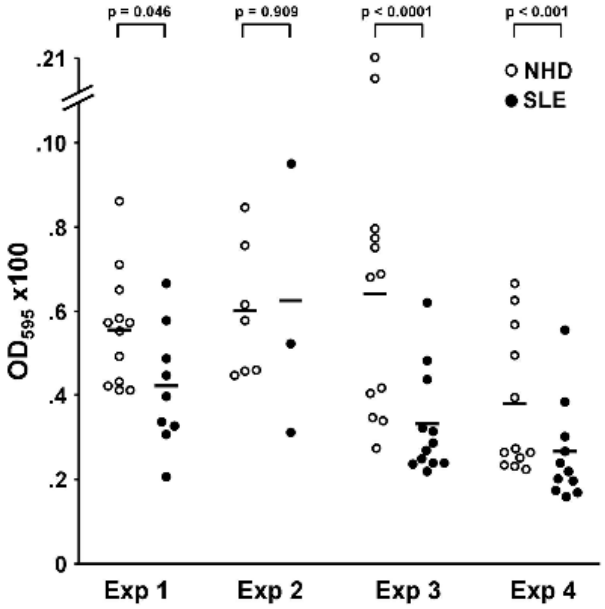


Fig. (2). Quantification of adhesion of cells differentiated from CFC. CFU were harvested and transferred into differentiation medium containing GM-CSF and FCS for one week. The differentiation of CFC into adherent cells was determined in four independent assays (Exp 1-4) and quantified by the crystal violet assay.

cells was impaired in 3 out of 4 patients with SLE when compared to healthy donors (Fig. **2**).

After culture in differentiation medium, adherent cells from healthy donors displayed a phenotype typical for active migrating cells with evident focal adhesions. Contrarily, cells from patients with SLE failed to display this phenotype. They were smaller, rounder, had very few focal adhesions, shorter pseudopodia, and were laxly attached to the plastic (Fig. **3**).

Expression of Macrophage Differentiation Markers on Peripheral Blood Hematopoietic Precursors and Stem Cell-Derived Cells

The frequency of circulating human CD34+/CD45+ stem cells in the PBMC population is very low and no difference between patients and controls was observed (0.06% ± 0.07% for healthy donors and 0.05% ± 0.04% for patients with SLE; p=0.7). After proliferation of the cells in methylcellulose-based medium, the phenotype of the descendants was determined by flow cytometry and is summarized in Fig. (**4**). CFC (Fig. **1A**) lost the expression of CD45 and CD34 during proliferation and express the adhesion molecule CD44, and the co-stimulatory membrane protein CD86. The phenotype of these CFC did not differ between patients and healthy donors. The differentiation of CFC of healthy donors into adherent cells (CFC-M) was characterized by the up-regulation of the integrin and receptor for complement and fibrinogen (CD11b), the receptor for LPS (CD14), and the low affinity receptor for IgG (CD16). Additionally, these cells recovered the expression of CD45 and retain CD86 on their surface. Contrarily, CFC-M derived from patients with SLE displayed a significant reduced expression of CD11b, CD14, CD45, and CD16 under the same conditions. MoMa served as a control population for the phenotype of mature phagocytes.

Phagocytosis of IgG Coated Beads by Stem Cell-Derived Adherent Cells is Impaired in SLE

Adherent cells derived from circulating stem cells were capable to phagocytose IgG opsonized beads after differentiation. Stem cell-derived adherent cells of healthy were filled with engulfed IgG opsonized beads. Otherwise, stem cell-derived adherent cells of patients with SLE often displayed a low content of engulfed IgG opsonized beads. Furthermore, much higher numbers of beads that were not taken up were observed in the cell cultures of the patients (Fig. **5**).

DISCUSSION

As common for the therapy of many chronic inflammatory diseases, the treatment of patients with SLE comprises the prevention of flares or reducing their severity and duration with the administration of high doses of corticosteroids, cytostatics, cyclosporine, and/or antibodies for longer periods of time [16]. All these drugs have profound effects on the biology of leukocytes and the immune system, even though after isolation of single cell populations *in vitro*. Defects in the recognition and engulfment of apoptotic cells

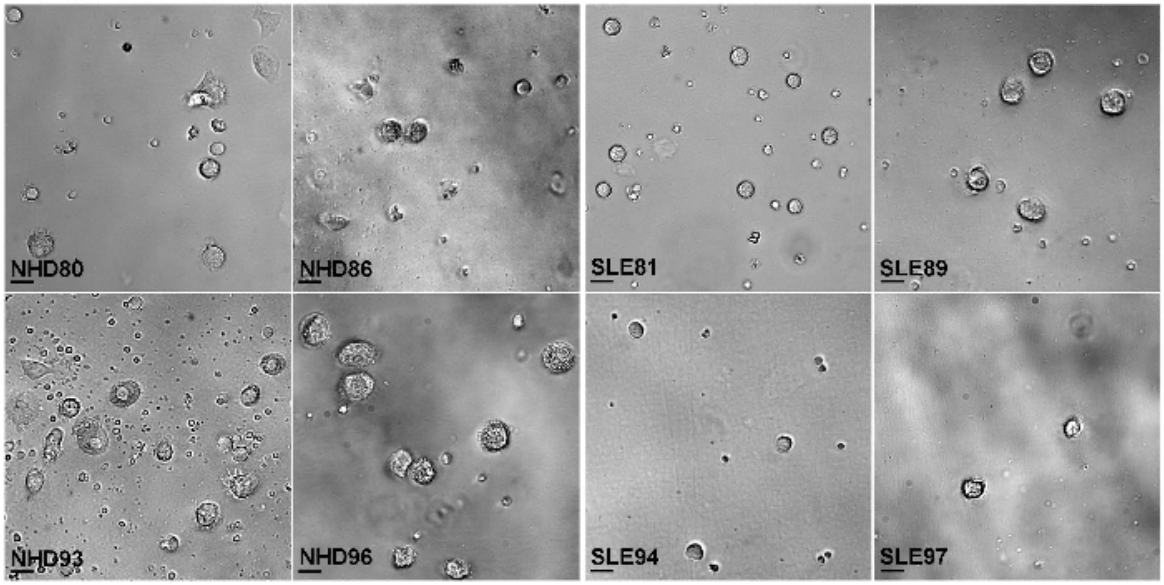
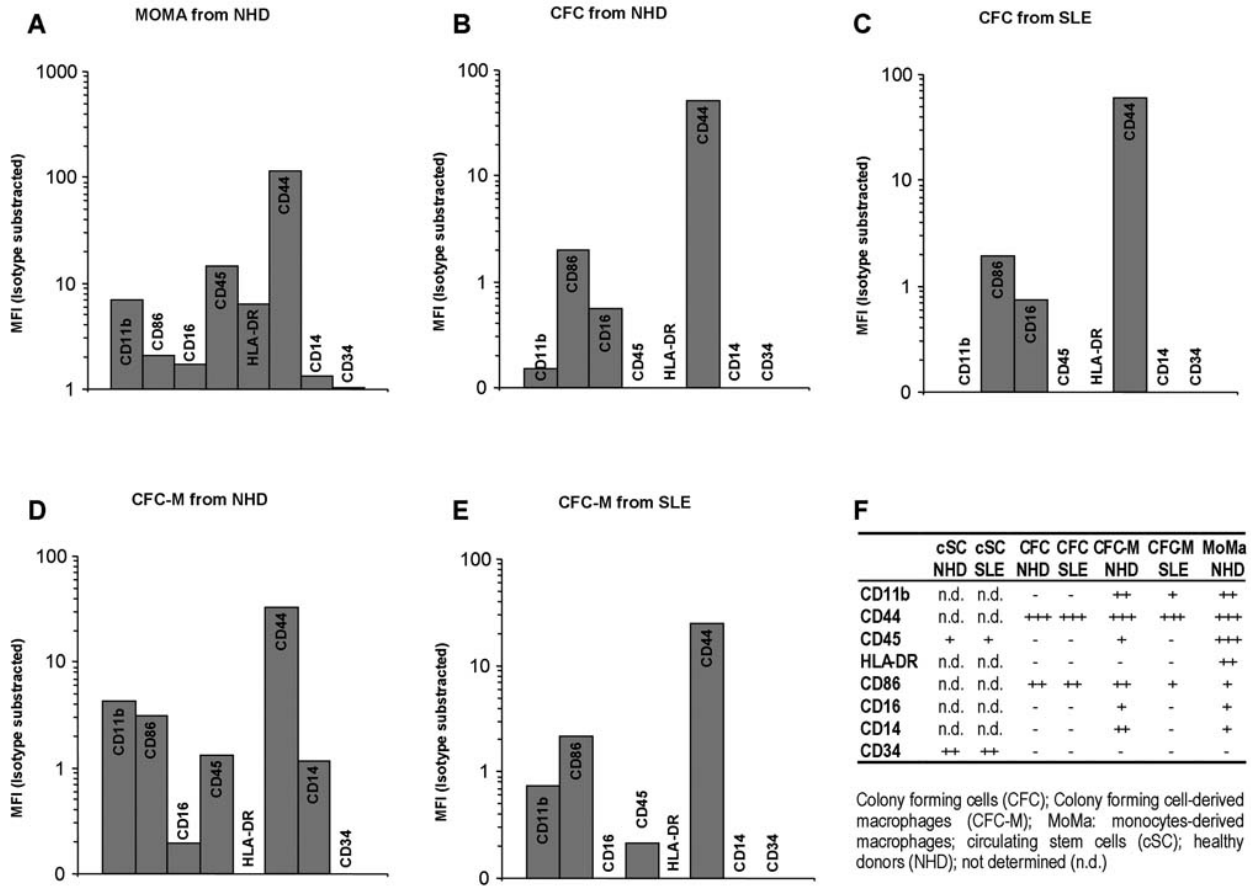


Fig. (3). Morphology of adherent cells differentiated from CFC. CFU were harvested and transferred into differentiation medium containing GM-CSF and FCS for one week. Adherent cells from healthy donors showed evident focal adhesions and displayed the morphology of macrophages, as examined by light microscopy. Contrarily, cells from patients with SLE failed to display the typical phenotype of active migrating cells; they were smaller and laxly attached to the plastic. Four representative set of images are displayed. The black bar represents 30 μm.



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	cSC NHD	cSC SLE	CFC NHD	CFC SLE	CFC-M NHD	CFC-M SLE	MoMa NHD
CD11b	n.d.	n.d.	-	-	++	+	++
CD44	n.d.	n.d.	+++	+++	+++	+++	+++
CD45	+	+	-	-	+	-	+++
HLA-DR	n.d.	n.d.	-	-	-	-	++
CD86	n.d.	n.d.	++	++	++	+	+
CD16	n.d.	n.d.	-	-	+	-	+
CD14	n.d.	n.d.	-	-	++	-	+
CD34	++	++	-	-	-	-	-

Colony forming cells (CFC); Colony forming cell-derived macrophages (CFC-M); MoMa: monocytes-derived macrophages; circulating stem cells (cSC); healthy donors (NHD); not determined (n.d.)

Fig. (4). Macrophage differentiation markers on hematopoietic precursors and their descendants. The indicated differentiation markers were quantified by flow cytometry on colony forming cells (CFC), colony forming cell-derived macrophages (CFC-M), monocytes-derived macrophages (MoMa), and circulating stem cells (cSC) from healthy donors (NHD) or patients with SLE. Representative mean fluorescence intensity values (MFI) corrected for isotype binding are displayed in Fig. (**4A-E**). The binding characteristics are summarized in (**F**); not determined (n.d.)

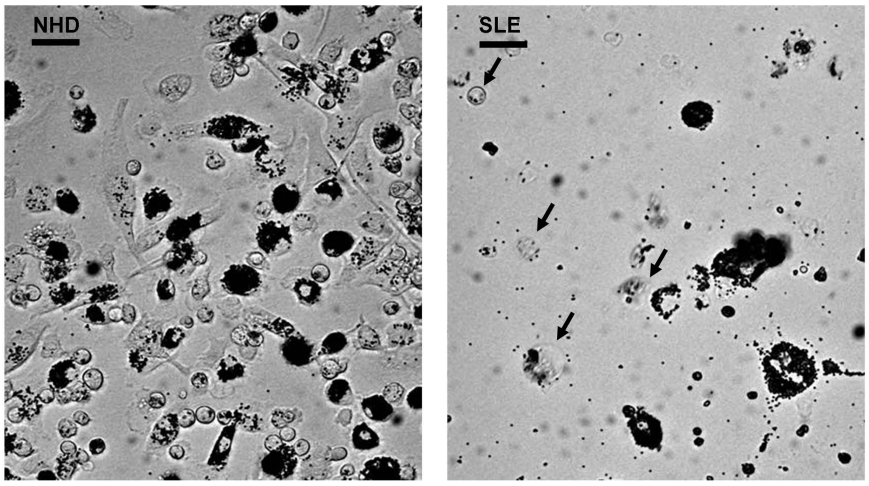

Fig. (5). Phagocytosis of IgG coated beads by stem cell-derived macrophages in healthy donors and patients with SLE. Macrophages from patients with SLE were scarce, smaller and were only round shaped, in comparison to those of healthy donors (NHD). Adherent cells with very low phagocytosis activity are marked with black arrows and were only present in the case of SLE. Furthermore, high numbers of non ingested beads (small black dots) were only seen in cell cultures obtained from cells of patients with SLE. One representative set of images out of five is displayed. The black bar represents 20 μm.

have been proposed being responsible for the accumulation of apoptotic cell-derived debris in patients with SLE [8, 17]. However, studies on phagocytes obtained from immune modulated patients will bear always the uncertainty about the influence of drugs on *in vitro* assays.

Adult stem cells can be expanded and differentiated *in vitro*. We used them to determine whether intrinsic defects contribute to the impaired clearance capabilities observed in SLE. Our studies revealed that circulating stem cells from patients with SLE had similar phenotype, frequency, viability, and proliferative potential as those from age and sex matched healthy donors. Many studies about the frequency of circulating SCs and its capacity to form progenitor colonies have been published. However, they differ considerably in the experimental settings. The frequency of circulating SCs in patients with SLE was lower than that of healthy donors when CD34 single staining [18] or CD34/CD133 double staining was employed [19]. Furthermore, the growth capability of circulating stem cells has been assessed in diverse conditioned media [19, 20]. We have cultured SCs in a methylcellulose base complete medium which foster the growth of different types of hematopoietic progenitors from single SCs. López-Karpovitch and collaborators employed a comparable medium to grow circulating SCs. However, they quantified CFU of granulocytes and monocytes only by cytochemical methods [20]. Our analysis of the cell cycle showed that circulating SCs from patients and controls are able to proliferate similarly in methylcellulose based medium to form colonies with different types of hematopoietic progenitors.

One of the main characteristic of monocytes and mature macrophages is their ability to adhere firmly to plastic and other surfaces. Adhesion has even been considered as phagocytosis of an infinitely large particle [21]. We used a colorimetric based method to quantify the amount of CFC which transformed under differentiation conditions into adherent cells. Much less adherent cells were observed in the case of SLE. Since the frequency of circulating SCs in patients and controls were similar, we suggest that hematopoietic precursors of patients with SLE have a reduced potential to differentiate into pleomorphic adherent cells. This may be due to an intrinsic impairment in the differentiation of SCs.

We further analyzed the phenotype of cells in each step of the differentiation process. We observed that descendants of circulating SCs from healthy donors and patients with SLE showed similar phenotype during proliferation and lacked the typical markers of mature macrophages. After differentiation, descendants of SCs from patients with SLE failed to up-regulate the macrophage markers CD11b, CD14, and CD16. On the basis of these observations we conclude that the differentiation program of circulating hematopoietic precursors of patients with SLE is intrinsically altered. This hypothesis is also supported by the observation that macrophages in the cultures of patients with SLE were scarce and displayed a lousy phagocytosis capability. We used beads for the phagocytosis assay to get first insights whether a modulation of this process takes place. Future work will focus in detail on various kinds of prey.

Taken together, we described in this work for the first time that SCs from patients with SLE are impaired to differentiate into adherent cells displaying the phenotype of mature macrophages. We suggest that the impaired clearance of apoptotic cells seen in patients with SLE may also involve intrinsic defects in the differentiation of myeloid progenitors. Such defects may explain the failure of the patients in clearing apoptotic cell debris from the sites of selection of autoreactive B cells.

ABBREVIATIONS

AAb = autoantibody

CFC = colony forming cells

CFC-M = CFC-derived adherent cells

CFU = colony forming unit

FCS = fetal calf serum

FITC = fluorescein isothiocyanate

GM-CSF = granulocyte-macrophage colony-stimulating factor

mAb = monoclonal antibody

MoMa = monocyte-derived macrophages

PBMC = peripheral blood mononuclear cells

PE = phycoerythrin

PI = propidium iodide

SC = stem cell

SLE = systemic lupus erythematosus

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