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Increased Expression of CD40 on Bone Marrow CD34+ Hematopoietic Progenitor Cells in Patients With Systemic Lupus Erythematosus

Contribution to Fas-Mediated Apoptosis

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Objective. **Patients with systemic lupus erythematosus (SLE) display increased apoptosis of bone marrow (BM) CD34+ hematopoietic progenitor cells. This study was undertaken to evaluate the expression of CD40 and CD40L in the BM of SLE patients, and to explore the possible involvement of these molecules in apoptosis of CD34+ cells.**

Methods. **The proportion and survival characteristics of CD40+ cells within the BM CD34+ fraction from SLE patients and healthy controls were evaluated by flow cytometry. The production of CD40L by BM stromal cells was assessed using long-term BM cultures, and the effect of CD40L on the survival characteristics and clonogenic potential of CD34+ cells was evaluated ex vivo by flow cytometry and clonogenic assays.**

Results. **SLE patients displayed an increased proportion of CD40+ cells within the CD34+ fraction as compared with controls. The CD34+CD40+ subpopulation contained an increased proportion of apoptotic cells compared with the CD34+CD40− fraction in patients and controls, suggesting that CD40 is involved in the apoptosis of CD34+ cells. Stimulation of patients' CD34+ cells with CD40L increased the proportion of apoptotic cells and decreased the proportion of colony-forming cells as compared with untreated cultures. The CD40L-mediated effects were amplified following treatment with recombinant Fas ligand, suggesting that the effects of these ligands are synergistic. CD40L levels were significantly increased in long-term BM culture supernatants and adherent layers of BM cells from SLE patients as compared with controls.**

Conclusion. **These data reveal a novel role for the CD40/CD40L dyad in SLE by demonstrating that upregulation and induction of CD40 on BM CD34+ cells from patients with SLE contribute to the amplification of Fas-mediated apoptosis of progenitor cells.**

The emerging role of hematopoietic and mesenchymal stem cells in the pathophysiology and treatment of autoimmune diseases has stimulated interest in the biologic properties of bone marrow (BM)–derived stem cells in these patients (1–4). In this context, we have previously shown that patients with systemic lupus erythematosus (SLE) display low numbers of BM CD34+ hematopoietic progenitor cells, due to induction of apoptosis by autoreactive T lymphocytes (5). These findings have been further substantiated by studies demonstrating low numbers of peripheral blood (PB) CD34+ cells in association with increased apoptosis (6), as well as abnormal immunophenotypic characteristics of BM CD34+ cells (7) in SLE patients. In addition, clinical data have shown a low PB CD34+ progenitor cell yield in SLE patients undergoing autologous stem cell transplantation, in comparison with that in patients with other autoimmune diseases (8).

Regarding the underlying pathophysiologic mecha-

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nisms, it has been shown that the Fas and Fas ligand (FasL) systems, belonging to the tumor necrosis factor (TNF) receptor (TNFR) and TNF families, respectively, is implicated, at least in part, in the apoptotic process of hematopoietic progenitor cells in SLE (5,7). Specifically, it has been suggested that increased local production of interferon-γ and FasL by autoreactive T lymphocytes results in up-regulation of Fas and apoptosis of CD34+ cells (9).

CD40, a TNFR family member, and its ligand, CD40L (CD154) (10), have also been implicated in the pathophysiology of SLE (11,12). It has been shown that overexpression of CD40L by activated T cells triggers a cascade of events in CD40+ target cells, resulting in humoral immune dysregulation and autoantibody production in SLE (12–15). Interestingly, a biologically active, membrane-cleaved, soluble form of CD40L (sCD40L) has been identified and found to be increased in patients' sera, which contributes to the pathophysiology of the disease by further augmenting the CD40-mediated signals (16–18).

The possible distribution of CD40 and its function on hematopoietic progenitor cells in SLE are, however, entirely unknown. It is of interest that although CD40–CD40L interactions have been associated with the rapid expansion of normal B lymphocytes, several studies have shown that the activation of CD40 in lymphoma, melanoma, carcinoma cells, and hepatocytes results in suppression of cell proliferation and/or induction of apoptosis (19–23). One of the mechanisms proposed to explain the proapoptotic function of CD40 in malignant cells and hepatocytes is the CD40-inducible up-regulation of Fas (23–26). However, whether such a cooperative interaction between the CD40 and Fas pathways is also implicated in the apoptosis of hematopoietic stem cells in SLE is unknown. Furthermore, although several studies have demonstrated increased amounts of circulating sCD40L in SLE, the cytokine levels in the SLE BM microenvironment and the possible pathophysiologic significance of these cytokines in SLE have so far not been investigated. Therefore, the present study was performed to evaluate the expression of CD40/CD40L in BM hematopoietic progenitor cells and the BM microenvironment in patients with SLE, and to explore the possible involvement of this receptor/ligand dyad in the apoptotic depletion of CD34+ cells.

PATIENTS AND METHODS

Patients. Twenty patients with SLE (19 women and 1 man; median age 43 years [range 19–73 years]) were enrolled in this study. All patients satisfied the American College of Rheumatology revised criteria for the diagnosis of SLE and had evidence of active disease according to the established criteria (27). The SLE Disease Activity Index (SLEDAI) score (scale 0–100) (28) was $\geq$4 in all patients. The characteristics of the patients are summarized in Table 1. Patients had discontinued any medication for at least 24 hours prior to BM aspiration. As controls, 20 hematologically healthy subjects, age- and sex-matched with the patients, were studied. Institutional ethics committee approval was granted prior to the study. Informed consent, in accordance with the Declaration of Helsinki, was obtained from all patients.

BM sample preparation. BM aspirates (10 ml) obtained from the posterior iliac crest of patients and healthy controls were immediately diluted 1:1 in Iscove's modified Dulbecco's medium (IMDM; Gibco Invitrogen, Paisley, Scotland) supplemented with 100 IU/ml penicillin–streptomycin (Gibco Invitrogen) and 10 IU/ml preservative-free heparin (Sigma-Aldrich, St. Louis, MO). Diluted BM samples were centrifuged on Histopaque 1077 (density 1.077 gm/cm^3; Sigma-Aldrich) at 400*g* for 30 minutes at room temperature to obtain BM mononuclear cells (BMMCs). Cell numbers and viability were assessed after staining with trypan blue.

Immunophenotyping and 7-aminoactinomycin D (7-AAD) staining. An indirect immunofluorescence technique was used to evaluate the expression of CD40 and Fas antigens within the BM CD34+ cell fraction. Briefly, 1×10^6 BMMCs were stained with phycoerythrin (PE)–conjugated mouse anti-human CD34 monoclonal antibody (mAb) (QBEND-10; Immunotech, Marseilles, France) and fluorescein isothiocyanate (FITC)–conjugated mouse anti-human CD40 (5C3; BD Biosciences-PharMingen, San Jose, CA) or Fas (CD95) (LOB 3/17; Serotec, Kidlington, UK) mAb. PE- and FITC-conjugated mouse IgG isotype-matched controls were used as negative controls. Following 30 minutes of incubation on ice, cells were washed twice in phosphate buffered saline (PBS)/1% fetal calf serum (FCS) (Gibco Invitrogen)/0.05% azide and were further stained with 7-AAD (Calbiochem-Novabiochem, La Jolla, CA) for the evaluation of the proportion of apoptotic cells, as previously described (29). Briefly, 100 μl 7-AAD solution (200 μg/ml) was added to the cells, followed by suspension in 1 ml PBS and incubation on ice for 20 minutes in the dark. Following centrifugation, the supernatant was removed, and cells were fixed in 500 μl 2% paraformaldehyde solution (Sigma-Aldrich). Unstained fixed cells were used as negative controls.

Cell samples were analyzed on an Epics Elite model flow cytometer (Coulter, Miami, FL) within 30 minutes of fixation. Data were acquired and processed on 500,000 events to evaluate the proportion of CD34+ cells within the BMMCs, the percentage of CD40+ and Fas+ cells in the CD34+ cell fraction, and the proportion of 7-AAD–negative (live), 7-AAD–dim (apoptotic), and 7-AAD–bright (dead) cells within the CD40- or Fas-expressing CD34+ cells.

Effect of CD40L on the survival characteristics and clonogenic potential of CD34+ cells. *Flow cytometric analysis.* CD34+ cells were isolated from the BMMCs of SLE patients and healthy controls by magnetic-activated cell sorting (MACS) analysis (MACS isolation kit; Miltenyi Biotec, Bergisch Gladbach, Germany) according to the manufacturer's protocol. The purity of the CD34+ cells was more than 96% in

Table 1. Clinical and laboratory characteristics of the patients with systemic lupus erythematosus*

Patient/ sex/age	Disease duration, years	SLEDAI	ANA titer	Anti-dsDNA	C3, mg/dl	Albumin, gm/dl	Hgb, gm/dl	Lymphocytes, $\times 10^9$/liter	Nephritis	CNS involvement	Previous medication
1/F/29	18	10	160	Positive	7.2	3.2	13.0	2.00	No	Yes	PDN, HCQ, CYC
2/F/43	8	6	320	Positive	90.4	3.9	7.1	1.25	No	No	PDN, HCQ, CYC
3/F/36	4	4	320	Positive	58.0	3.2	9.5	1.25	No	No	None
4/F/39	2	12	1,280	Positive	66.2	3.5	13.7	1.40	Yes	Yes	PDN, HCQ, CYC
5/F/58	8	23	160	Positive	112.0	4.3	13.9	2.10	Yes	Yes	HCQ
6/F/43	5	19	3,200	Negative	94.0	3.2	12.5	0.50	Yes	No	PDN
7/F/46	3	4	80	Negative	137.0	4.1	10.9	2.40	No	No	HCQ
8/F/19	4	4	640	Negative	89.4	4.4	13.0	1.10	Yes	No	PDN, HCQ, MMF
9/F/62	13	2	80	Negative	104.0	4.0	14.6	2.90	No	No	HCQ, CYC
10/F/36	12	10	160	Negative	51.0	3.5	11.8	1.20	No	No	HCQ
11/F/59	15	4	1,280	Negative	123.0	5.4	11.6	0.50	No	Yes	PDN, CYC
12/M/73	1	11	160	Negative	106.0	5.6	12.9	0.80	No	No	PDN, CYC
13/F/43	4	19	160	ND	58.0	3.5	12.0	1.20	No	No	HCQ
14/F/48	2	16	640	Negative	111.0	4.3	13.9	4.00	No	Yes	PDN, HCQ
15/F/57	9	7	640	Negative	58.0	3.6	10.2	0.60	Yes	No	PDN, HCQ, CYC
16/F/44	8	14	320	ND	51.0	3.5	14.7	1.20	No	No	PDN, HCQ, CYC
17/F/22	4	9	1,280	Positive	78.6	3.8	10.9	0.30	Yes	No	PDN, CYC
18/F/26	7	9	320	Positive	51.0	3.5	9.5	0.90	Yes	No	PDN, HCQ, CYC
19/F/28	12	19	320	Positive	89.0	3.8	9.5	1.10	No	No	None
20/F/60	13	7	160	Negative	58.0	3.8	11.3	1.50	Yes	No	HCQ, CYC

* SLEDAI = Systemic Lupus Erythematosus Disease Activity Index (scale 0–100); ANA = antinuclear antibody; anti-dsDNA = antibodies against double-stranded DNA; Hgb = hemoglobin; CNS = central nervous system; PDN = prednisolone; HCQ = hydroxychloroquine; CYC = cyclophosphamide; MMF = mycophenolate mofetil; ND = not determined.

all experiments. We then cultured CD34+ cells in 96 round-bottomed well plates at a density of 1×10^4 CD34+ cells/well in the presence of 1 μg/ml recombinant human CD40L (rHuCD40L) (BMS308/2; Bender MedSystems, Vienna, Austria) and/or 1 μg/ml rHuFasL (BMS309/2; Bender MedSystems) in 200 μl IMDM/1% FCS. Following 48 hours of incubation, the cells were stained with 7-AAD in the same manner as described above, for the evaluation of the proportion of live, apoptotic, and dead CD34+ cells. In a number of experiments, Fas antigen expression was also evaluated on CD34+ cells that had been treated in the same manner as described above, with 1 μg/ml rHuCD40L.

Clonogenic assay. We cultured 3×10^3 CD34+ cells from SLE patients and normal controls in 1 ml IMDM supplemented with 30% FCS, 1% bovine serum albumin (BSA) (Gibco Invitrogen), $10^{-4}M$ mercaptoethanol (Sigma-Aldrich), 0.075% sodium bicarbonate (Gibco Invitrogen), 2 m*M* L-glutamine (Sigma-Aldrich), and 0.9% methylcellulose (StemCell Technologies, Vancouver, British Columbia, Canada), in the presence of 5 ng granulocyte–macrophage colony-stimulating factor (R&D Systems, Minneapolis, MN), 50 ng interleukin-3 (R&D Systems), and 2 IU erythropoietin (Janssen-Cilag, Bucks, UK) in the presence or absence of 1 μg rHuCD40L and/or 1 μg rHuFasL. Cultures were set up in duplicate in 35-mm petri dishes and incubated at 37°C in a fully humidified atmosphere of 5% CO_2. In a separate set of experiments, 10^5 BMMCs were cultured as described above, in the presence or absence of 1 μg/ml CD40L neutralizing antibody (MK13A4; Bender MedSystems) or with the same quantity of PBS as a control. In all cases, on day 14 of the cultures, colonies were scored as previously described, and results were expressed as the total number of colony-forming cells (CFCs) (30).

Long-term BM cultures (LTBMCs). For preparation of LTBMCs, 10^7 BMMCs were grown according to a standard technique (30), in 10 ml IMDM supplemented with 10% FCS, 10% horse serum (Gibco Invitrogen), 100 IU/ml penicillin–streptomycin, 2 m*M* L-glutamine, and $10^{-6}M$ hydrocortisone sodium succinate (Sigma-Aldrich), and incubated at 33°C in a fully humidified atmosphere of 5% CO_2. At weekly intervals, cultures were examined for stromal layer formation, using an inverted microscope, and were fed by removing half of the medium and replacing it with an equal volume of fresh IMDM supplemented as described above. At weeks 3–4, when a confluent stromal layer was formed, cell-free supernatants were stored at −72°C for the determination of sCD40L, using a commercially available (Bender MedSystems) enzyme-linked immunoabsorbent assay (ELISA). The sensitivity of the assay is 0.6 ng/ml. The culture samples were tested by ELISA after being concentrated using a Speedvac method (31).

For the assessment of CD40L expression in LTBMCs of stroma, total messenger RNA (mRNA) was extracted from the adherent cells of confluent LTBMCs, using the RNeasy mini kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. Contaminating DNA was removed by digestion with RNAse-free DNAse. The SuperScript Preamplification System (Gibco Invitrogen) was used for first-strand complementary DNA (cDNA) synthesis from 1 μg total RNA, followed by reverse transcription–polymerase chain reaction (PCR) with specific primers. PCR products were normalized according to the amount of β_2-microglobulin (β_2m) in the samples. We performed one tube reaction, and the primer

Table 2. Flow cytometric analysis of BMMCs in SLE patients and healthy controls*

	SLE patients (n = 20)	Healthy controls (n = 20)	*P*
BMMC cell fraction, CD34+ cells			
%	1.14 ± 0.35	2.06 ± 0.62	<0.0001
Median (range) number	1.10 (0.70–1.90)	1.75 (1.30–3.30)	
CD34+ cell fraction			
Fas+ cells			
%	20.69 ± 19.53	7.16 ± 4.46	0.0138
Median (range) number	15.95 (1.30–79.00)	6.70 (1.60–16.10)	
CD40+ cells			
%	10.79 ± 6.90	5.41 ± 2.89	0.0061
Median (range) number	11.10 (3.80–33.00)	5.20 (0.70–12.00)	
7-AAD^{dim} cells			
%	26.03 ± 26.40	4.47 ± 2.21	<0.0001
Median (range) number	17.40 (3.00–87.10)	3.90 (2.20–80.60)	
CD34+Fas+ cell fraction, 7-AAD^{dim} cells			
%	43.50 ± 24.56	25.60 ± 14.15	0.0084
Median (range) number	52.60 (1.20–86.40)	22.20 (6.00–50.00)	
CD34+Fas− cell fraction, 7-AAD^{dim} cells			
%	22.89 ± 28.38	3.24 ± 2.25	0.0066
Median (range) number	12.80 (0–87.80)	2.20 (1.20–7.60)	
CD34+CD40+ cell fraction, 7-AAD^{dim} cells			
%	37.43 ± 18.24	23.07 ± 10.03	0.0051
Median (range) number	37.50 (1.60–84.00)	20.50 (9.10–44.40)	
CD34+CD40− cell fraction, 7-AAD^{dim} cells			
%	11.85 ± 9.72	5.41 ± 6.41	0.2134
Median (range) number	10.00 (0–25.90)	2.50 (1.30–19.80)	

* Except where indicated otherwise, values are the mean ± SD. BMMC = bone marrow mononuclear cell; SLE = systemic lupus erythematosus; 7-AAD^{dim} = 7-aminoactinomycin D–dim (apoptotic).

concentration of CD40L was 7 times higher than the concentration of the β_2m primer.

The forward and reverse primers were 5′-AGAATCCTCAAATTGCGGC-3′ and 5′-TGTGGGTATTTGCAGCTCTG-3′, respectively, for CD40L (PCR product size 286 bp), and 5′-TCCAACATCAACATCTTGGT-3′ and 5′-TCCCCCAAATTCTAAGCAGA-3′, respectively, for β_2m (PCR product size 123 bp). Conditions for 37 cycles of PCR amplification following initial denaturation were 94°C for 30 seconds, 62°C for 30 seconds, and 72°C for 45 seconds. PCR products were electrophoresed on a 1.5% agarose gel and visualized under ultraviolet light by ethidium bromide staining. The positive control was cDNA from the Ms5-CD40L–transfected cell line (provided by one of us [AGE]). Results were analyzed using the ImageJ analysis program (National Institutes of Health, Bethesda, MD).

Statistical analysis. Data were analyzed using the GraphPad Prism statistical program (GraphPad Software, San Diego, CA) by means of the nonparametric Mann-Whitney and Spearman's tests. Student's *t*-test for paired samples was used to compare differences in the proportion of Fas+ cells and the number of CFCs between cultures treated and those not treated with rHuCD40L or CD40L neutralizing antibody. Group data are expressed as the mean ± SD.

RESULTS

Expression of CD40 and Fas antigens on CD34+ cells. Results from flow cytometric analysis of the CD34+ cells from patients with SLE are presented in Table 2. In accordance with previously reported data (5), the proportion of CD34+ cells within the BMMC fraction was statistically significantly lower in SLE patients (mean ± SD 1.14 ± 0.35%) compared with healthy controls (2.06 ± 0.62%; $P < 0.0001$), and the proportion of Fas+ cells within the CD34+ cell compartment was significantly higher in patients (20.69 ± 19.53%) compared with controls (7.16 ± 4.46%; $P = 0.0138$). The percentage of CD40-expressing CD34+ cells was significantly increased in SLE patients (10.79 ± 6.90%) compared with healthy controls (5.41 ± 2.89%; $P = 0.0061$). Moreover, a highly significant correlation was found between the proportion of CD40-expressing CD34+ cells and the proportion of Fas-expressing CD34+ cells (r = 0.5358, $P = 0.0004$), suggesting a parallel mode of expression of the 2 molecules in the study cell population (Figure 1). Notably, no statistically significant difference was found between patients and controls in the proportion of CD40+ cells detected in the non-CD34+ BMMC fraction (7.03 ± 2.65% and 6.13 ± 2.43%, respectively; $P = 0.818$).

Survival characteristics of the CD34+CD40+ cells. Data on the survival characteristics of the patients' CD34+ cells are presented in Table 2. In accordance with the results from previously reported studies (5), the

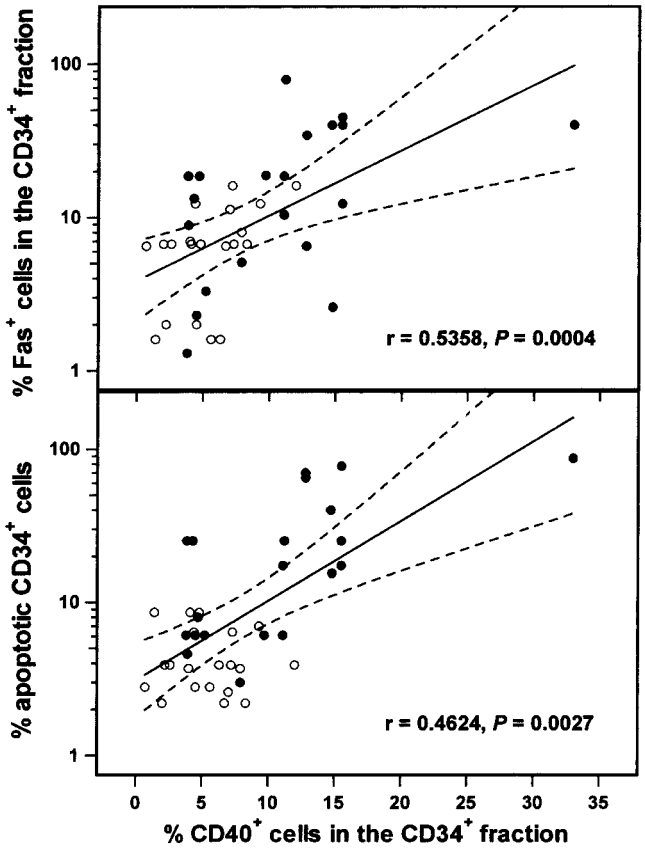

Figure 1. Correlations between the proportion of CD40+ cells and the percentages of Fas+ cells **(top)** and apoptotic cells **(bottom)** within the CD34+ cell fraction of bone marrow from patients with systemic lupus erythematosus (n = 20; solid circles) and healthy subjects (n = 20; open circles), as determined by linear regression analysis. Regression lines and the 95% confidence intervals are shown as solid lines and broken lines, respectively.

proportion of apoptotic cells within the CD34+ compartment was significantly increased in SLE patients (26.03 ± 26.40%) compared with healthy controls (4.47 ± 2.21%; $P < 0.0001$). The percentage of apoptotic cells was higher in the CD34+Fas+ cell compartment compared with the CD34+Fas− cell compartment, in both the SLE patients (43.50 ± 24.56% versus 22.89 ± 28.38%; $P = 0.0098$) and the healthy controls (25.60 ± 14.15% versus 3.24 ± 2.25%; $P < 0.0001$), whereas no significant difference was documented in the proportion of apoptotic cells detected in the Fas+ and Fas− subpopulations of the non-CD34+ cell compartment, in either the patients (2.87 ± 2.60% and 3.74 ± 3.97%, respectively; $P = 0.6848$) or the controls (1.89 ± 2.07% and 1.62 ± 1.35%, respectively; $P = 0.7452$). These findings further highlight the previously reported prominent role of the Fas antigen in the apoptosis of BM CD34+ cells (5,32,33). Interestingly, an increased rate of apoptosis was also observed in the CD34+Fas− cell fraction in the patients compared with the controls ($P = 0.0066$), suggesting that additional mechanisms other than up-regulation of Fas antigen expression are operating in the apoptotic depletion of CD34+ cells in SLE.

To investigate the possible involvement of CD40 in the apoptotic process of CD34+ cells, we studied the survival characteristics of the CD40+ and CD40− cell subpopulations. In the gate of CD34+ cells, the proportion of apoptotic cells was significantly higher among the CD40+ cells than among the CD40− cells, in both the patients (37.43 ± 18.24% versus 11.85 ± 9.72%; $P < 0.0001$) and the controls (23.07 ± 10.03% versus 5.41 ± 6.41%; $P < 0.0001$) (Table 2). In the gate of CD34− cells, however, no statistically significant difference in the percentage of apoptotic cells was documented between the CD40+ and CD40− cell subpopulations, in either the patients (2.40 ± 2.42% versus 3.30 ± 3.65%; $P = 0.5792$) or the controls (2.47 ± 1.80% versus 1.65 ± 1.64%; $P = 0.638$). Furthermore, among all of the subjects studied, a highly significant correlation was noted between the proportion of CD40+ cells and the percentage of apoptotic cells within the CD34+ subpopulation (r = 0.4624, $P = 0.0027$) (Figure 1), but not within the non-CD34+ subpopulation (r = 0.1887, $P = 0.818$). These results indicate that overexpression of the CD40 antigen may have a role in the apoptotic depletion of BM CD34+ cells in patients with SLE.

All of the patients studied had active disease. However, CD40 expression on CD34+ cells did not correlate with parameters of disease activity, such as the SLEDAI score, albumin levels, antinuclear antibody titers, and C3 values. Nevertheless, there was a correlation with hemoglobin levels (r = −0.6689, $P = 0.0013$), further indicating a possible apoptosis-inducing role of CD40 on hematopoiesis in patients with SLE.

Effect of CD40L on CD34+ hematopoietic progenitor cell death. To investigate the hypothesis that the CD40 antigen may directly and/or indirectly have a role in the apoptotic process of CD34+ cells, we evaluated the proportion of apoptotic cells following incubation of purified CD34+ cells from SLE patients (n = 3) and healthy controls (n = 3) with rHuCD40L and/or rHuFasL. The results are presented in Figure 2A. In SLE patients, the addition of rHuFasL significantly increased the proportion of apoptotic cells (33.93 ± 3.52%) compared with untreated (baseline) cultures (24.27 ± 3.78%; $P = 0.0317$). The addition of rHuCD40L also

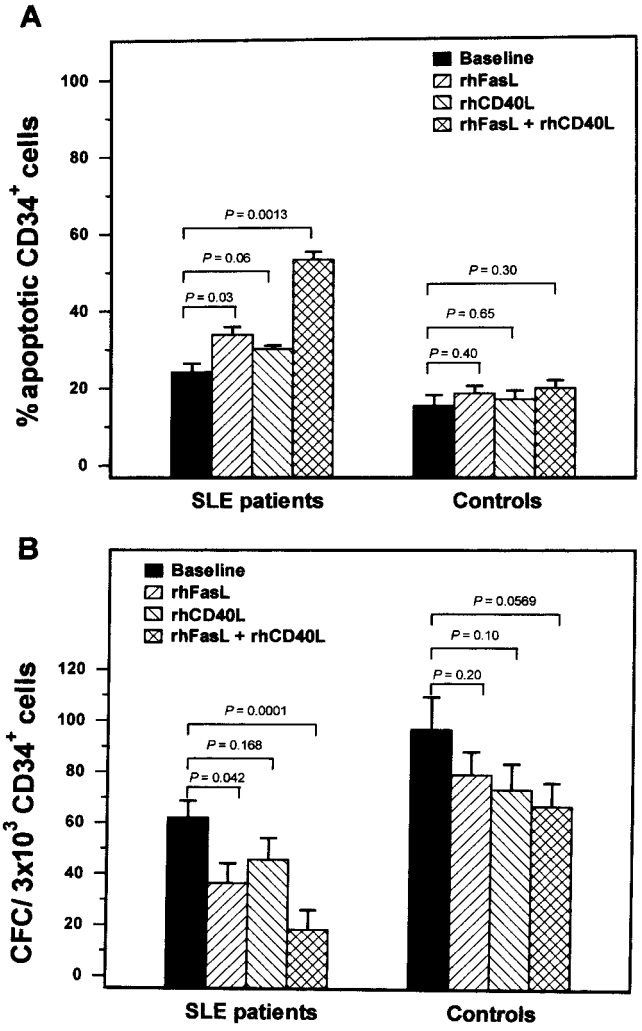

Figure 2. Effect of CD40L on the survival characteristics and clonogenic potential of CD34+ cells. **A,** Proportion of apoptotic CD34+ cells in the bone marrow of patients with systemic lupus erythematosus (SLE) (n = 3) and healthy subjects (n = 3) following 48-hour incubation of the cells with recombinant human Fas ligand (rhFasL) (1 μg/ml) and/or recombinant human CD40L (1 μg/ml). Apoptosis was assessed by flow cytometry and 7-aminoactinomycin D staining. **B,** Numbers of colony-forming cells (CFCs) per 3×10^3 CD34+ cells, determined using a clonogenic assay, in patients with SLE (n = 11) and healthy subjects (n = 11) following 14-day culture of the cells with recombinant human FasL (1 μg/ml) and/or recombinant human CD40L (1 μg/ml). In both series of experiments, treated cultures were compared with untreated cultures (baseline) using the nonparametric Mann-Whitney test. Bars show the mean and SD.

increased the proportion of apoptotic cells (30.20 ± 1.47%), but not to a statistically significant extent as compared with untreated cultures ($P = 0.0645$). The addition of both rHuCD40L and rHuFasL resulted in a statistically significant increase in the proportion of apoptotic cells compared with that in untreated cultures (53.26 ± 11.23%; $P = 0.0013$), compared with that in cultures treated with rHuFasL alone ($P = 0.0466$), or compared with that in cultures treated with rHuCD40L alone ($P = 0.0024$). In healthy controls, the addition of rHuCD40L and/or rHuFasL did not result in statistically significant increases in the proportion of apoptotic cells as compared with that in untreated cultures, probably because of the low levels of expression of surface CD40 and Fas on normal CD34+ cells.

These data suggest that CD40L displays an indirect, rather than a direct, apoptosis-inducing effect on CD40-expressing CD34+ cells, by facilitating the FasL-mediated apoptotic process. To substantiate this hypothesis, we evaluated Fas antigen expression on immunomagnetically sorted CD34+ cells from SLE patients (n = 12) following a 48-hour incubation with rHuCD40L. We found that the proportion of Fas+ cells significantly increased in cultures treated with rHuCD40L (48.60 ± 12.63%) as compared with that in untreated cultures (23.71 ± 7.47%; $P < 0.0001$). The up-regulation of Fas expression by CD40L may therefore explain, at least in part, the amplifying effects of CD40L on Fas-mediated CD34+ cell death.

Effect of CD40L on the clonogenic potential of CD34+ cells. To further characterize the effect of CD40L on the function of CD34+ cells, we investigated the clonogenic potential of CD34+ cells from SLE patients (n = 11) and healthy controls (n = 11) in the presence of rHuCD40L and/or rHuFasL. Results are depicted in Figure 2B.

In cultures of patients' cells treated with rHuFasL, a significant decrease in CFC number (36.54 ± 25.46 CFCs per 3×10^3 CD34+ cells) was observed in comparison with that in untreated cultures (62.09 ± 22.09 CFCs per 3×10^3 CD34+ cells; $P = 0.0418$). In the presence of rHuCD40L, CFC numbers were similarly decreased (45.73 ± 28.16 CFCs per 3×10^3 CD34+ cells) as compared with baseline; however, the difference was not statistically significant. A profound decrease in CFC number was observed in the presence of both rHuCD40L and rHuFasL (18.45 ± 12.10 CFCs per 3×10^3 CD34+ cells) as compared with that in untreated cultures ($P = 0.0001$) or compared with that in cultures treated with rHuCD40L alone ($P = 0.0256$) or rHuFasL alone ($P = 0.0459$). Clonogenic assays using CD34+ cells from healthy subjects demonstrated that rHuCD40L and/or rHuFasL reduced the number of CFCs as compared with that in untreated cultures. However, these differences were not statistically significant. Collectively, these data suggest that CD40L exerts,

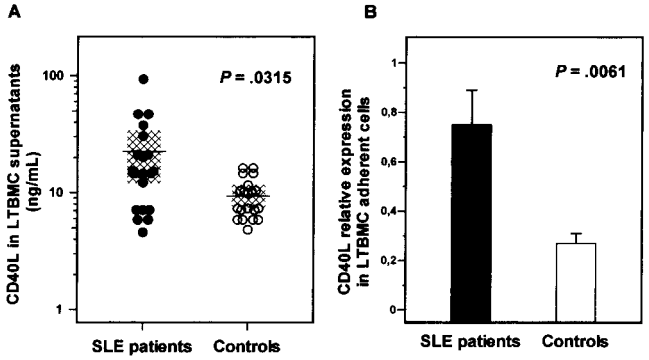

Figure 3. Concentration of CD40L in long-term bone marrow cultures (LTBMCs). **A,** Cells from SLE patients (n = 20) and healthy controls (n = 20) were harvested at confluence, and levels of CD40L, upon concentration in LTBMC supernatants, were measured by enzyme-linked immunosorbent assay. The mean concentration and 95% confidence intervals are indicated by horizontal lines and shaded areas, respectively. **B,** CD40L mRNA expression was determined in LTBMC adherent layers of cells from all SLE patients and healthy controls. Bars show the mean and SD cumulative data. Groups were compared using the nonparametric Mann-Whitney test. See Figure 2 for other definitions.

mainly, a synergistic effect on the FasL-induced impairment of the clonogenic potential of CD40-expressing CD34+ cells, rather than a direct negative effect.

Increased CD40L expression in SLE LTBMCs. To probe the pathophysiologic significance of the increased expression of CD40 on CD34+ cells from patients with SLE, we evaluated CD40L levels and CD40L mRNA expression in LTBMCs, an experimental design that represents an in vitro model mimicking the BM microenvironment (30). The levels of sCD40L were first determined in LTBMC supernatants. Results are presented in Figure 3A.

SLE patients displayed increased sCD40L levels (21.97 ± 21.07 ng/ml) as compared with healthy controls (9.42 ± 3.63 ng/ml; $P = 0.0315$), suggesting that local production of sCD40L is increased in the BM of SLE patients. Individual sCD40L levels inversely correlated with the proportion of CD34+ cells in the subjects studied ($r = -0.6139, P < 0.0001$), further indicating the negative effect of sCD40L on the reserves of BM CD34+ cells in SLE patients.

To further substantiate the increased local production of CD40L in SLE BM, we evaluated the cytokine mRNA expression in LTBMC adherent cell extracts from patients and healthy controls. Cumulative data are shown in Figure 3B. Consistent with the ELISA data shown in Figure 4A, CD40L mRNA expression was significantly increased in SLE patients (0.75 ± 0.62 ng/ml) as compared with that in healthy controls (0.27 ± 0.18 ng/ml; $P = 0.0061$).

Increase in clonogenic potential of SLE BM progenitor cells by neutralization of CD40L. To further substantiate the involvement of the CD40 pathway in BM progenitor cell survival in SLE patients, we evaluated the clonogenic potential of patient and normal BMMCs in the presence or absence of a CD40L neutralizing antibody. On the basis of our observations indicating that CD40 was overexpressed on the patients' CD34+ cells in the BMMC fraction (Table 2) and that CD40L was overproduced by cells in the BM microenvironment (Figure 3), it was anticipated that neutralization of CD40L would increase the CFC number selectively in SLE patient samples, but not in the controls.

Indeed, neutralization of CD40L in BMMCs

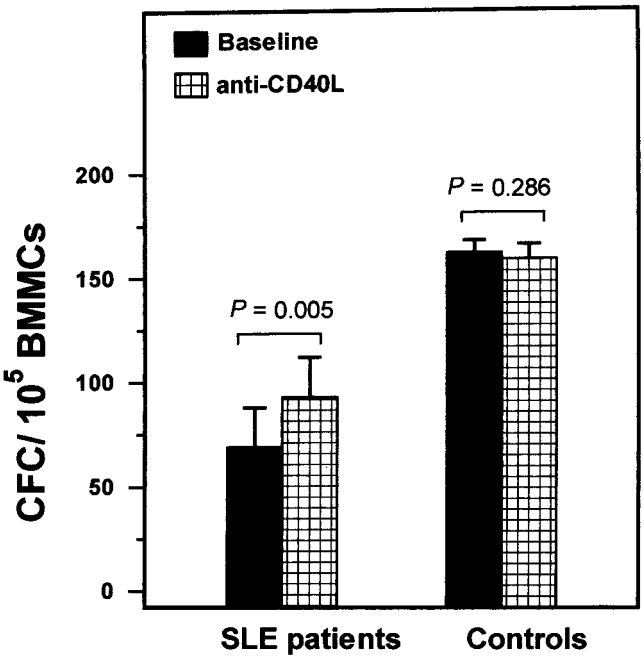

Figure 4. Effects of neutralization of CD40L on the clonogenic potential of bone marrow progenitor cells from patients with SLE. Bone marrow mononuclear cells (BMMCs) (10^5) from SLE patients (n = 6) and healthy controls (n = 6) were cultured using a standard clonogenic assay, in 1 ml Iscove's modified Dulbecco's medium supplemented with 30% fetal calf serum, 1% bovine serum albumin, $10^{-4}M$ mercaptoethanol, 0.075% sodium bicarbonate, 2 m*M* L-glutamine, 0.9% methylcellulose, 5 ng granulocyte–macrophage colony-stimulating factor, 50 ng interleukin-3, and 2 IU erythropoietin in the presence or absence of 1 μg CD40L neutralizing antibody. Bars show the mean and SD number of CFCs obtained in SLE patients and controls in antibody-treated and untreated (baseline) cultures. Groups were compared using Student's *t*-test for paired samples. See Figure 2 for other definitions.

obtained from SLE patients (n = 6) resulted in a statistically significant increase in the clonogenic potential of BMMCs (93 ± 47 CFCs per 10^5 BMMCs) as compared with that in cultures that did not receive anti-CD40L antibody (70 ± 48 CFCs per 10^5 BMMCs; $P = 0.005$) or that in cultures with the same quantity of PBS (69 ± 46 CFCs per 10^5 BMMCs; $P = 0.003$). In contrast, neutralization of CD40L had no impact on the clonogenic potential of BMMCs obtained from control subjects (n = 6) (159 ± 16 CFCs per 10^5 BMMCs versus 162 ± 17 CFC per 10^5 BMMCs in untreated cultures; $P = 0.286$) (Figure 4). Thus, our findings suggest that CD40L is functionally expressed in BMMCs.

DISCUSSION

There is increasing evidence that immune dysregulation in patients with SLE may disturb the morphology and function of the BM microenvironment and may severely affect BM hematopoiesis (9,34). Autoreactive lymphocytes and proinflammatory mediators have been primarily implicated in BM damage, in that these elements suppress the hematopoiesis-supporting capacity of BM stromal cells and accelerate the apoptosis of hematopoietic stem/progenitor cells (5). A varying degree of "unexplained" cytopenias, extreme cases of aplastic anemia, and insufficient CD34+ cell yield upon mobilization are abnormalities attributed to the immune-mediated damage of BM in SLE (35,36). A better understanding of the mechanisms that induce the stem/progenitor cell damage in SLE would have major clinical implications in SLE, in view of the current interest in exploring the use of autologous stem cells in the treatment of severely affected patients (37), since elucidating the effects of these cells may explain potential problems in the mobilization, harvesting, and marrow repopulating procedures.

In the present study, we have investigated the involvement of CD40–CD40L interactions in the apoptotic process of the BM hematopoietic stem/progenitor cells in patients with SLE. Although these 2 TNFR/TNF family members are aberrantly expressed in patients' PB lymphocytes and serum, they have never been studied in the BM. We found that CD40 was minimally expressed on normal hematopoietic progenitor cells; however, the proportion of CD40-expressing CD34+ cells was significantly increased in SLE patients. In accordance with our previously reported findings on the reserves and survival characteristics of BM CD34+ cells in SLE (5), we found that SLE patients had a significantly lower percentage of CD34+ cells in the BMMC fraction and displayed an increased proportion of apoptotic, Fas-expressing cells within the CD34+ cell compartment as compared with the the levels in healthy subjects.

To explore the possible association of CD40 up-regulation with the apoptotic depletion of the hematopoietic progenitor cells in SLE patients, we performed a subset analysis of the survival characteristics of CD34+ cells on the basis of CD40 surface expression. We found that the proportion of apoptotic CD34+ cells was significantly increased in the CD40+ cells as compared with the CD40− cell fraction, in both patients and healthy controls, suggesting a possible involvement of CD40 in the apoptotic process of BM hematopoietic progenitor cells. In favor of this hypothesis was the strong correlation between the proportion of CD40+ cells and the percentage of apoptotic CD34+ cells in the study population. In contrast, CD40 antigen expression did not affect the survival characteristics of cells in the CD34− BMMC fraction, which comprises mainly the lymphocyte subsets (11).

CD40 ligation appears to have opposing effects on cell viability, depending on the cell target (11). Interestingly, CD40-dependent apoptotic processes have been reported to be mediated via up-regulation of other TNFR family molecules, including Fas (22,24). Moreover, a cooperative interaction between CD40 and Fas in the promotion of the apoptotic process has been demonstrated in human hepatocytes (23), B lymphoma cells (38), and carcinoma cells (24). Our data demonstrating that incubation of patients' CD34+ cells with CD40L resulted in up-regulation of surface Fas indicate, for the first time, that a cooperative mechanism between the CD40 and Fas pathways also exists in BM CD34+ cells.

In accordance with previously reported data (5), SLE patients were found to display an increased proportion of Fas+ cells in the CD34+ BMMC fraction, and incubation of purified CD34+ cells with FasL resulted in a significant increase in the proportion of apoptotic cells, suggesting that functional Fas expression takes place in the BM. The strong correlation between the proportion of CD40- and Fas-expressing CD34+ cells in SLE patients indicates a possible cooperative mode of action of these molecules in accelerating progenitor cell death. Evidence to support this hypothesis comes from our observation that ex vivo treatment of patients' CD34+ cells with the combination of CD40L and FasL resulted in amplification of apoptosis as compared with that in cultures exposed to each ligand alone.

It is of interest that treatment of patients' CD34+ cells with CD40L alone resulted in a low level of apoptosis induction, suggesting that CD40 activation per se

may affect CD34+ cell survival. However, a number of observations suggest that CD40 activation confers predominantly indirect negative effects on the survival of BM progenitor cells through the amplification of Fas-induced apoptosis. Thus, exposure of patients' CD34+ cells to rHuCD40L resulted in up-regulation of Fas expression. Moreover, we have shown that in vitro exposure to a combination of CD40L and FasL induced a profound reduction in the number of CFCs in SLE patients as compared with treatment with each cytokine alone, and that the relative reduction in CFC numbers was higher following FasL treatment as compared with that following CD40L treatment.

It has been suggested that elevated levels of sCD40L in SLE patient sera, in association with increased and prolonged expression of the membrane form of the molecule on patients' T cells, may contribute to the pathophysiology of the disease by amplifying the aberrant activation signals through the target cell CD40 (16). In our study, we evaluated sCD40L in LTBMC supernatants of patients' cells, reflecting the cytokine levels within the BM microenvironment. We found that culture supernatant levels of sCD40L were significantly increased in patients compared with controls, and that these levels correlated inversely with the proportion of CD34+ cells. This finding coupled with the observed CFC recovery following CD40L neutralization in patient, but not control, BMMC cultures further highlight the negative effect that the CD40 pathway may exert on hematopoiesis in patients with SLE. Consistent with the elevated sCD40L levels in the supernatants was the increased cytokine mRNA expression in LTBMC adherent layers derived from SLE patients compared with that in normal LTBMCs. The cellular origin of CD40L in the BM microenvironment in SLE is unknown. CD40L might be produced by a variety of cells that constitute the LTBMC adherent layers, such as monocyte/macrophages, endothelial cells, and fibroblasts (11), but also might be produced by lymphocytes that persist in LTBMC conditions (9,39).

In summary, this study is the first to evaluate the expression of CD40/CD40L in the BM progenitor cell microenvironment in patients with SLE and healthy subjects. Our data reveal a novel role for CD40 in BM homeostasis in SLE, by demonstrating that up-regulation of the CD40 pathway amplifies Fas-mediated CD34+ hematopoietic progenitor cell death and contributes to defective BM progenitor cell reserve and function, both of which are characteristic of SLE. These results further substantiate the rationale of CD40/CD40L blockade as treatment of SLE, not only to reduce autoantibody production but also to ameliorate cytopenias associated with BM dysfunction. The intracellular pathways and signaling cascades involved in this intriguing crosstalk between CD40 and Fas, the possible implication of other TNF/TNFR family members, as well as the effect of the treatment on CD40 expression are interesting areas for further investigation.

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AUTHOR CONTRIBUTIONS

Dr. Papadaki 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 design. Eliopoulos, Papadaki.

Acquisition of data. Pyrovolaki, Mavroudi, Sidiropoulos, Boumpas, Papadaki.

Analysis and interpretation of data. Eliopoulos, Papadaki.

Manuscript preparation. Eliopoulos, Boumpas, Papadaki.

Statistical analysis. Papadaki.

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