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Bone Marrow Clonogenic Capability, Cytokine Production, and Thymic Output in Patients with Common Variable Immunodeficiency[1]

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In patients with primary Ab deficiencies, hematological and immunological abnormalities are frequently observed. A regenerative failure of hemopoietic stem/progenitor cells has been hypothesized. We evaluated in the bone marrow (BM) of 11 patients with common variable immunodeficiency, the phenotype of BM progenitors and their in vitro growth by colony-forming cell (CFC) and long-term culture (LTC) assays. A significant decrease in erythroid and mixed CFC and, to a greater extent, in primitive LTC-CFC progenitors was observed in patients compared with healthy controls. The frequency of BM pre-B and pro-B cells correlated directly with the absolute number of $CD19^+$ lymphocytes. BM cells cultured in vitro produced spontaneously lower amounts of IL-2 and elevated levels of TNF-α compared with controls, indicating a skewing toward a proapoptotic cytokine pattern. In addition, stromal cells generated after BM LTC secreted less IL-7 and displayed by immunohistochemistry an altered phenotype. These findings were associated with a significant decrease in naive Th cells coexpressing CD31 in the peripheral blood. These results indicate an impaired growth and differentiation capacity of progenitor cells in patients with common variable immunodeficiency. *The Journal of Immunology,* **2005, 174: 5074–5081.**

Common variable immunodeficiency (CVID)[3] represents an heterogeneous group of disorders characterized by decreased serum level of Igs and recurrent bacterial infections, mainly of the respiratory and gastrointestinal tracts (1). In these patients, an increased prevalence of autoimmune disorders, granulomatous diseases, neoplasias, and malnutrition has been observed (2–4).

Thrombocytopenic purpura and hemolytic anemia represent the most frequent autoimmune disorders (23), but nonautoimmune cytopenias are also common in patients with CVID (5). Although the association between cytopenias and CVID is unclear, the defects in T cell regulation (6, 7), cytokine production (8, 9), abnormal apoptosis (10), and abnormal Ig secretions (11) are all potential mechanisms involved in the pathogenesis of these disorders.

In approximately one-third of patients with CVID, T cells are also decreased as absolute number, with an inversion of the CD4:CD8 ratio due to reduced $CD4^+$ T cells, especially those expressing the $CD4^+CD45RA^+$ (naive) phenotype, whereas the number of B cells is variable (normal to very low) (12–15), often with a deficiency in memory B cells along with somatic hypermutation (16). To explain these findings, several hypotheses have been proposed, including deficient IL-2 production (17) and/or increased T cell apoptosis due to the persistent Ag activation after infections (10). However, the production of T cell precursors in CVID patients was never investigated in detail. Thymic activity may be ineffective in replenishing the pool of peripheral T lymphocytes. Recently, TCR excision circles (TREC) frequencies in peripheral blood of CVID patients have been investigated and a significant diminished content of TREC levels both in $CD4^+$ and in $CD8^+$ T compartments, compared with those of age-matched healthy controls has been observed (18). These results suggest a more rapid reduction of thymic output in CVID individuals, but it remains to be clarified whether the damage of the T cell compartment is related to an altered generation of new T cells from hemopoietic stem/progenitor cells or to a defect in thymic and/or bone marrow (BM) epithelial cell compartment.

In vivo development of lymphoid progenitors requires a strict interaction of these cells with the BM stromal microenvironment that provides a rich milieu of cytokines, extracellular matrix proteins, and adhesion molecules (19, 20). Analysis of BM and stromal cytokine production may be important in understanding the causes of the hematological abnormalities occurring in CVID patients. Impaired stromal function, alteration of hemopoietic growth factor network, and abnormal apoptosis may be all involved in impaired hematolymphopoiesis of these patients.

At present, no data are available on BM functions, because the literature reports are limited to sporadic cases with identified hematological abnormalities.

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[3] Abbreviations used in this paper: CVID, common variable immunodeficiency; TREC, TCR excision circle; BM, bone marrow; CFC, colony-forming cell; LTC, long-term culture; BMMC, bone marrow mononuclear cell; LTBMC, long-term BM cultures; IVIG, i.v. Ig; rhu, recombinant human; CFU-GEMM, CFU-granulocyte-erythrocyte-monocyte-megakaryocyte; BFU-E, burst-forming unit-erythroid; CFU-E, CFU-erythroid; CFU-GM, CFU-granulocyte-monocyte; LTC-IC, LTC-initiating cell.

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To investigate the BM function in patients with CVID, we analyzed the BM progenitor cell growth and differentiation with colony-forming cell (CFC) and long-term culture (LTC)-CFC assays, the spontaneous cytokine production (IL-2 and TNF-α) at BM level, the BM stromal cell morphology, and the stromal IL-7 production. In addition, we analyzed, as thymic naive Th cells (21), the coexpression of $CD31^+$ in peripheral $CD45RA^+$ $CD4^+$ T cells to extend the previous data about thymic output in CVID patients (18).

Materials and Methods

Patient population

The study group consisted of 11 patients with CVID, followed as outpatients at the Division of Allergy and Clinical Immunology, University of Rome "La Sapienza" (Rome, Italy) and recently at Interregional Center for Primary Immunodeficiencies (Rome, Italy). They were selected on a voluntary basis for BM aspirates and enrolled in this study. Two of them presented with leukopenia and thrombocytopenia. All subjects gave their written informed consent for the breastbone aspiration, according to the Ethical Committee procedures at our institution. The main clinical and immunological characteristics of the patients are reported in Table I. The mean age ranged from 22 to 59 years (42 ± 12). The diagnosis was made according to the criteria of the WHO experts' group for primary immunodeficiency diseases (1). All men underwent the B tyrosine kinase test with negative results (data not shown). Five CVID patients received regular substitution therapy with i.v. Ig (IVIG) at the standard dose of 400 mg/kg body weight every 3 wk from several years. The duration of the disease was 8.3 ± 8 years. At time of breastbone aspiration, six subjects, just diagnosed as CVID patients, were naive for IVIG treatment. To assess the patients' clinical conditions, a scoring system has been elaborated, and the following parameters were collected retrospectively during the previous 3 years: presence (=1) or absence (=0) of three or more of severe bacterial infections and/or recurrent less severe infectious episodes. None of the patients had symptoms of acute inflammatory disease or elevated levels of C-reactive protein in the serum at the time of the study. There was no evidence of autoimmune disorders, granulomatous diseases, or neoplasias in any patient. At the time of the BM investigation, none of the patients had acute infection.

As controls, 10 healthy donors matched by sex and age with the patients were included. Eight of ten subjects also underwent BM aspirates (all had normal BM). None of the controls had acute infections and/or received medications at the time of the study.

Serum Ig analyses

Serum concentrations of IgG, IgM, and IgA were measured by immunonephelometry (Behring 100), using N antisera to human Igs (IgG, IgM, and IgA) (Dade Behring Marburg). Normal laboratory ranges for serum Igs were 690-1400 mg/dl for IgG, 70–370 mg/dl for IgA, and 40–240 mg/dl for IgM.

Flow cytometric analysis of PBMCs

Whole blood phenotype analysis consisted of lysing 500 μl of blood with 10 ml of Ortho-mune Lysing Reagent (Ortho Diagnostic Systems) at room temperature, washing, and labeling with a mixture of four mAbs for 30 min at 4°C. Anti-CD3-FITC, anti-CD4-allophycocyanin, anti-CD8-PerCP, anti-CD45RA-FITC, anti-CD62L-PE, and anti-CD19-PE were purchased from BD Biosciences. In nine CVID patients, to identify thymic naive Th cells, we used the following Abs as previously described (21): anti-CD4-PerCP, CD45RA-FITC, and anti-CD31-PE, all from BD Biosciences. After staining, cells were washed once in PBS containing 2% FBS and analyzed on a FACSCalibur cytofluorometer (BD Biosciences) using CellQuest software. Absolute lymphocyte counts were calculated by standard hemocytometric technique.

Preparation of BM mononuclear cells (BMMCs)

BM aspirates were initially collected into a tube containing EDTA as anticoagulant. The BM samples were diluted 1/3 with 1× PBS plus 5 mM EDTA and then separated after centrifugation by Ficoll (Lymphoprep; Nycomed Pharma) and resuspended in RPMI 1640 supplemented with 10% FCS, L-glutamine (2 mmol/L), and penicillin (250 U/ml) (all from Invitrogen Life Technologies).

Flow cytometry of BMMCs

Flow cytometric analysis was performed with freshly collected BM samples from patients or normal BM donors. Briefly, 1×10^6 BMMCs were washed twice with 1× PBS and then labeled with the following fluorochrome-conjugated mAbs: anti-CD34-PerCP; anti-CD45-FITC; anti-CD38-PE; anti-CD19-PE; anti-CD10-FITC; and anti-HLA-DR-FITC (BD Biosciences). $CD34^+$ cells were defined as positive events in the low side scatter region, $CD34^+CD45^{low}$ windows; progenitor cell subpopulations were calculated within the $CD34^+CD45^+$ gate, expressed as relative percentages of the $CD34^+$ cells.

CFC assay and long-term BM cultures (LTBMC)

BMMCs (1×10^5) were plated in duplicate cultures in 1 ml of methylcellulose assay medium containing recombinant human (rhu) erythropoietin (3 U/ml), rhu stem cell factor (50 ng/ml), rhu-GM-CSF (10 ng/ml), and rhu IL-3 (10 ng/ml) (BIOPSA; Stem Cell Technologies). In addition, in three CVID patients, we analyzed the effects of anti-TNF-α and of cytokines IL-7, IL-2, and IL-2 plus IL-7 on BM colony formation by CFC assay. Briefly, 1×10^5 BMMCs were plated in duplicate cultures in 1 ml of methylcellulose assay medium with or without anti-TNF-α (1 μg/ml), IL-7 (20 ng/ml), IL-2 (10 ng/ml), or IL-2 plus IL-7 (10 and 20 ng/ml, respectively) (all from R&D Systems). According to standardized morphological criteria, the growth of the multipotent hemopoietic progenitor was evaluated as CFU-granulocyte-erythrocyte-monocyte-megakaryocyte (CFU-GEMM), burst-forming unit-erythroid (BFU-E), CFU-erythroid (CFU-E), and CFU-granulocyte-monocyte (CFU-GM) after 2 wk of incubation at 37°C in an atmosphere of 5% CO_2.

To analyze the most immature progenitors and the stem cell compartment, we used LTBMC. Stromal cell cultures and determination of LTC-CFC numbers were performed according to a modification of the described methods (22). The murine BM stromal cell line M210B4 was cultured until cell confluence and then trypsinized, irradiated (8000 rad), washed, and placed in six-well plates. Total BMMCs (1×10^6 cells, in duplicate cultures) were applied on the pre-established, irradiated stromal feeder layers and cultured at 37°C for 5 wk. In three CVID patients, BMMCs were seeded on the stromal feeder layers and cultured with the weekly addition

Table I. *Clinical, serological, and hematological characteristics of CVID patients at enrolment for BM aspirates*[a]

Patients	Sex	Age (yr)	Score	IgGa	IgA	IgM	IgGb	Hb (g/dl)	PTL ($10^3/\mu$l)	WBC ($10^3/\mu$l)	Lymph (cells/μl)	$CD4^+$ (cells/μl)	$CD8^+$ (cells/μl)	$CD4^+CD8^+$	$CD19^+$ (cells/μl)
1. D.D.	M	38	1	183	<4.3	<5.8	*[b]	14.6	120	4.5	630	296	195	1.5	16
2. G.C.	M	34	1	100	<4.3	<5.8	553	12	66	2.3	650	234	247	0.9	6.5
3. S.M.	F	38	1	209	21	20	*	11.2	212	6.1	1016	417	224	1.9	264
4. Z.G.	M	55	0	150	<4.3	<5.8	*	9.9	57	1.7	451	153	271	0.6	14
5. P.E.	M	22	0	460	20	17	*	16	189	4.6	994	278	298	0.9	99
6. M.T.	F	53	1	310	<4.3	26	607	15.4	385	13.6	5900	1652	3363	0.5	354
7. G.L.	F	59	1	300	<4.3	28	714	11.4	133	4.9	955	257	420	0.6	134
8. C.R.	M	51	1	188	<4.3	<5.8	626	13.3	156	8.0	1902	361	1065	0.3	0
9. M.G.	M	44	1	230	<4.3	<5.8	*	15.3	182	6.9	1519	289	714	0.4	182
10. G.R.	F	58	1	290	<4.3	<5.8	701	12	73	1.7	774	248	348	0.7	89
11. S.W.	F	64	1	129	<4.3	18	*	12.9	168	6.1	1723	982	362	2.7	68
Control (Mean ± SD)	5M/5F	40 ± 11	0	690–1400	88–410	34–210		12.4 ± 0.7	291 ± 27	7.9 ± 1.4	2156 ± 432	1024 ± 162	404 ± 178	2.6 ± 1.3	157 ± 53

[a] To assess patient's clinical conditions a scoring system has been elaborated, and the following parameters were collected retrospectively during a 3-year follow-up: presence (=1) or absence (=0) of three or more severe bacterial infections and/or recurrent less severe infectious episodes. IgG[a], IgA, and IgM values (milligrams per deciliter) before starting IVIG treatment; IgGb values (milligrams per deciliter) after IVIG treatment (at time of breastbone aspiration).

[b] * Naive for IVIG treatment.

Table II. *Analysis of $CD4^+$ and $CD8^+$ T cells and the coexpression of CD31 in peripheral naive $CD45RA^+$ Th cells*

	Patients										
	1. D.D.	2. G.C.	5. P.E.	6. M.T.	7. G.L.	8. C.R.	9. M.G.	10. G.R.	11. S.W.	CVID (mean ± SD)	Control (n = 10; mean ± SD)
$CD4^+$ (%)	47	36	28	28	27	19	19	32	57	34 ± 12*[a]	47.5 ± 6
$CD4^+$ (cells/μl)	296	234	278	1652	257	361	289	248	982	351 ± 233**	1024 ± 162
$CD4^+$naive[b] (%)	4	6.8	25.8	4	11.6	7.9	14	12	2	10 ± 7**	52 ± 12
$CD4^+$naive (cells/μl)	12	16	72	66	30	29	40	30	20	31 ± 19**	519 ± 131
$CD4^+CD45RA^+CD31^+$ (%)	2	5	22	3	9	8	12	9	1.5	8 ± 6**	37 ± 10
$CD4^+CD45RA^+CD31^+$ (cells/μl)	6	12	61	50	23	29	35	22	15	25 ± 17**	385 ± 50
$CD8^+$ (%)	31	38	30	57	44	56	47	45	21	39 ± 16**	20 ± 7
$CD8^+$ (cells/μl)	195	247	298	3363	420	1065	714	348	362	414 ± 272	404 ± 178
$CD8^+$naive (%)	25	30	43.9	7	16.9	12.9	20	13	15	22 ± 11**	58 ± 15
$CD8^+$naive (cells/μl)	49	74	131	235	71	138	143	45	54	88 ± 12**	233 ± 100

[a] * $p = 0.01$; **, $p \leq 0.002$.
[b] Naive, $CD45RA^+CD62L^+$.

of anti-TNF-α (1 μg/ml), IL-7 (20 ng/ml), or IL-2 (10 ng/ml) to evaluate their effect on immature progenitor cells.

The half-medium liquid was changed weekly. After 5 wk, the nonadherent and adherent cells were harvested by treatment with trypsin (Invitrogen Life Technologies), washed, and replaced in duplicate in methylcellulose to evaluate the number of cells able to determine secondary colonies. The number of CFC generated after 5 wk of cultures on stromal cells gives an indirect but consistent measurement of the content of LTC-initiating cells (LTC-IC) (22).

Spontaneous cytokine production from BMMC cultures

To evaluate the cytokine production at BM level, BMMC short-term cultures were performed with freshly collected BM samples from CVID patients, as well as from normal BM donors. Briefly, isolated BMMCs were cultured in RPMI 1640 supplemented with 10% FCS, L-glutamine (2 mmol/L), and penicillin (250 U/ml) in a 5% CO_2 atmosphere at 37°C, in the absence of stimuli to verify the spontaneous production of cytokines IL-2 and TNF-α. After 24 h of culture, supernatants were collected, and cytokines were measured by ELISA, according to the manufacturer's instructions (R&D Systems). For each cytokine determination, a standard curve was set up. In addition BMMCs (1×10^6/well), obtained from three CVID patients and two controls, were cultured, and the supernatants were collected after 24, 48, 72, and 96 h for IL-2 or TNF-α time kinetic study by ELISA.

BM stromal cells characterization by immunohistochemistry

BMMCs were cultured in tissue culture chamber slides (Falcon) in IMDM (Invitrogen Life Technologies) supplemented with 20% FCS, 100 IU/ml penicillin-streptomycin, and 100 IU/ml glutamine at 37°C in humidified air at 5% CO_2. At weekly intervals, cultures were fed by demipopulation of the nonadherent cells and replacement of 500 μl of fresh supplemented IMDM. The cultures were maintained until stromal confluence (3–4 wk) and then analyzed by immunohistochemistry. Stromal cells were fixed with acetone-ethanol (50:50) for 30 min and then incubated for 30 min with the following primary Abs: anti-CD68 (1/200) and anti-CD14 (5/100) as macrophage markers; anti-CD34 (1/25); as progenitor cell marker; anti-S100 (1/1000) as adipocyte marker; and anti-vimentin (1/50) as mesenchymal marker (all from DAKO; Dakopatts). These samples were then stained to amplify and detect the signal, according to the manufacturer's instructions, using an immunoperoxidase technique (DAKO LSAB Kit-peroxidase; Dakopatts).

The slides were subsequently analyzed using light microscopy.

Spontaneous IL-7 production from BM stromal cultures

BMMCs obtained from seven CVID patients and three healthy subjects were cultured in 24-well plates in IMDM (Invitrogen Life Technologies), supplemented with 10% FCS, 10% horse serum, 100 IU/ml penicillin-streptomycin, 100 IU/ml glutamine, and 10^{-6} M hydrocortisone sodium succinate (Sigma-Aldrich), at a concentration of 1×10^6 cells/ml in a total volume of 2 ml/well. The plates were incubated at 37°C in humidified air at 5% CO_2.

At weekly intervals, cultures were fed by demipopulation of the nonadherent cells and replacement of 500 μl of fresh, supplemented IMDM. The cultures were maintained until stromal confluence (3–4 wk); then the cells were collected by trypsinization and cultured at a concentration of 1×10^6 cells/ml in a total volume of 1 ml/well. Supernatants were then collected after 24 h of culture and cytokine IL-7 was measured by ELISA, according to the manufacturer's instructions with an ultrasensitive kit (R&D Systems).

Statistical analysis

Nonparametric statistics were used (Mann-Whitney, Wilcoxon test) for unpaired and paired comparisons between the parameters analyzed in patients and healthy individuals. A simple regression test was used to correlate the group characteristics. A value of $p < 0.05$ and $R > 0.5$ or < -0.5 were considered significant. Statistical analyses were performed with Stat View 5.0 software (SAS Institute).

Results

Patients' features

Patients showed pretherapy a mean value of serum IgG of 224 ± 105 mg/dl, generally associated with low or undetectable IgA and

Table III. *Evaluation of percentage of BMMCs by flow cytometry in CVID patients vs controls*

Patients	$CD34^+CD45^{+a}$	$CD34^+CD38^+DR^{+b}$	$CD34^+CD38^-DR^{+b}$	$CD34^+CD19^+CD10^{+b}$	$CD34^-CD10^{+a}$
1	5.6	93	2	11.6	4.8
2	1.7	94	1.7	4.3	0.5
3	2.4	91	1.5	26	11
4	0.5	89	1.9	1	0.5
5	1.3	91	0	15	3.3
6	0.5	71	3	58	0.4
7	1.5	94	0.2	11	2
8	1	86	1	0	0.09
9	3	89	3.3	3	9
10	5	85	2	5	10
11	2	85	2.5	10	4.5
CVID (mean ± SD)	2.5 ± 1.7	89.7 ± 3.5	1.6 ± 1	8.7 ± 7.8	4.6 ± 4
Control (n = 8) (mean ± SD)	2.6 ± 1.5	88 ± 7.3	5.6 ± 1.2	15.9 ± 7.9	5.1 ± 0.8

[a] Within the lymphocyte/blast gate.
[b] Within the $CD34^+CD45^+$ gate.

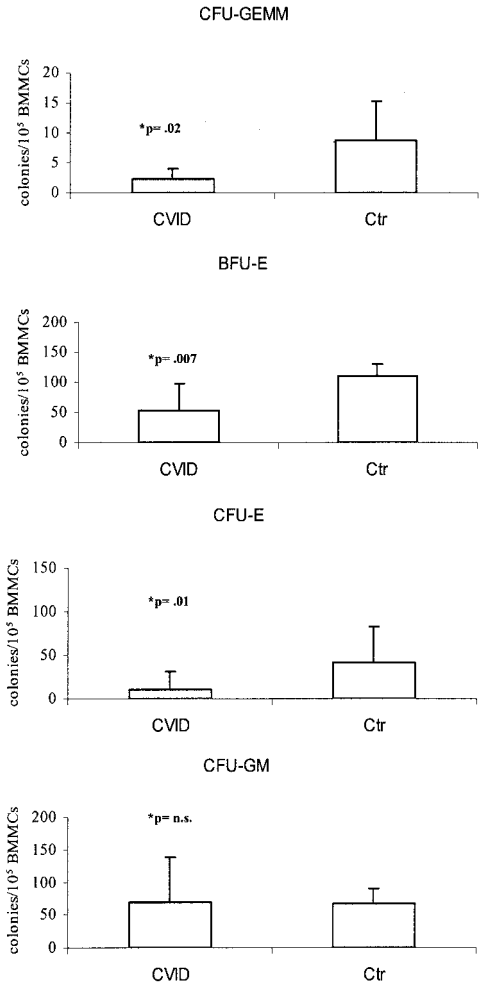

FIGURE 1. Clonogenic progenitor cells in CVID patients. Bars, Mean number (±SD) of colonies obtained by 100,000 BMMCs plated in methylcellulose (CFC assay) from 11 CVID patients and 8 healthy controls (Ctr). The growth of CFU-GEMM, BFU-E, CFU-E, and CFU-GM was evaluated according to standardized morphological criteria.

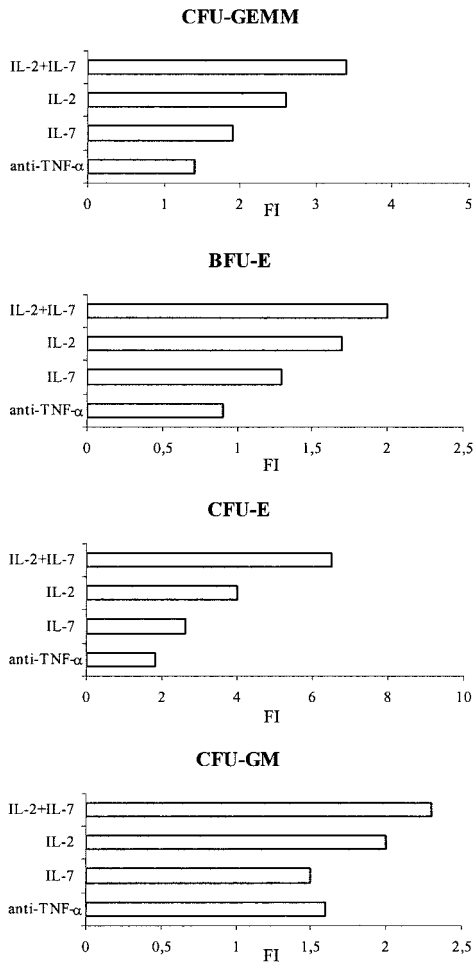

FIGURE 2. Effect of anti-TNF-α, IL-7, or IL-2 on CFC assays in CVID patients. Bars, Mean fold increase (FI; with respect to the values without growth factors) of colonies obtained by 1×10^5 BMMCs plated in methylcellulose (CFC assay) from three CVID patients with the addition of anti-TNF-α (1 μg/ml), IL-7 (20 ng/ml), IL-2 (10 ng/ml), or IL-2 plus IL-7 (10 and 20 ng/ml, respectively).

IgM serum levels (Table I). According with the scoring system, some subjects had three or more infectious episodes per year despite IVIG treatment (see Table I). No one showed altered results of liver and kidney functions or a positivity for hepatitis B virus surface Ag and/or presented hepatitis C virus infection. The Dixon test was negative in all patients with thrombocytopenia.

Immunological and hematological data in PBMCs

Hematological and immunological data of the patients are reported in detail in Tables I and II.

Patients had significantly lower $CD4^+$ T cells than did the controls (351 ± 233 cells/μl vs 1024 ± 162 cells/μl, respectively; $p = 0.0007$), and this reduced number was observed mainly in $CD45RA^+CD62L^+$ (naive phenotype) subset (10 ± 7% in patients vs 52 ± 12% in controls; $p = 0.0004$). In addition, in nine CVID patients, we observed a significant decrease of peripheral $CD45RA^+CD31^+$ Th cells (thymic naive Th cells) (on average 8 ± 6% in patients vs 37 ± 10% in controls; $p = 0.0004$). $CD8^+$ T cell numbers did not differ statistically between patients and controls (414 ± 272 cells/μl vs 404 ± 178 cells/μl; p NS), but the percentage of $CD8^+$ naive T cells was significantly lower in patients (22 ± 11% vs 58 ± 15%; $p = 0.0007$). The CD4:CD8 T cell ratio was reduced (<0.75) in 6 of 11 patients (patients 4, 6, 7, 8, 9, and 10). Seven patients (patients 1, 2, 4, 5, 8, 10, and 11) displayed reduced numbers of B cells vs the control subjects, whereas three patients (n. 1, 2 and 8) had only 0–1% of $CD19^+$ cells.

Hematological abnormalities were observed: decreased hemoglobin levels (<12 g/dl) in three patients (patients 3, 4, and 7), low white blood cell counts ($<4.0 \times 10^3$ cells/μl) in three patients (patients 2, 4, and 10), and thrombocytopenia ($<150 \times 10^3$ cells/μl) in five patients (patients 1, 2, 4, 7, and 10).

Flow cytometric BMMCs analysis

Table III shows the results of the flow cytometric BM assessment. The mean proportion of $CD34^+$ cells in BMMCs was in the range of normal controls, although in three patients (patients 4, 6, and 8) $CD34^+$ cells were ≤1%. The percentage of phenotypically primitive $CD34^+CD38^-DR^+$ cells was 1.6 ± 1 (vs 5.6 ± 1.2% in normal subjects). The proportion of $CD34^+$ cells expressing CD19 and CD10 (pro-B cells) was lower than that of the controls (8.7 ± 7.8% vs 15.9 ± 7.9%) and five patients (patients 2, 4, 8, 9, and 10) displayed very low levels of this progenitor cell subset (≤5%). $CD34^-CD10^+$ cells (pre-B lymphocytes) did not differ from the normal range (4.6 ± 4% vs 5.1 ± 0.8%), despite the fact that eight

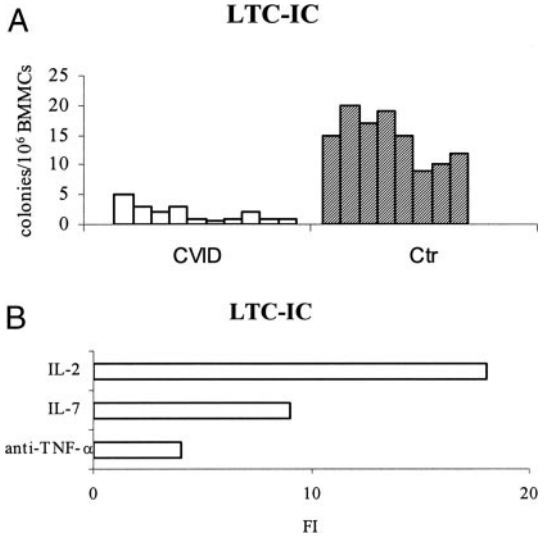

FIGURE 3. LTC-IC assay in CVID patients. *A,* Bars, number of colonies obtained by LTC-IC assay from 11 CVID patients (□) and 8 healthy controls (Ctr; ■). Total BMMCs (1×10^6 cells, in duplicate cultures) were applied on a pre-established, irradiated stromal feeder layer and cultured at 37°C for 5 wk. Cells were then harvested and plated in a methylcellulose assay to evaluate the number of cells able to form secondary colonies (see *Materials and Methods*). *B,* Bars, Fold increase (FI; with respect to the values without growth factors) of colonies obtained by 1×10^6 BMMCs cultured from three CVID patients, with the addition of anti-TNF-α (1 μg/ml), IL-7 (20 ng/ml), or IL-2 (10 ng/ml).

patients (patients 1, 2, 4, 5, 6, 7, 8, and 10) showed pre-B cell percentages <5%. A direct correlation was also observed between the numbers of BM pro-B cells ($p = 0.003$, $R = 0.677$), pre-B cells ($p = 0.01$, $R = 0.760$), and peripheral blood $CD19^+$ lymphocytes.

CFC assay and LTBMC

As reported in Fig. 1, the number of CFC was lower in BM from patients than in BM from control subjects, with specific regard to erythroid and mixed progenitors (11.2 ± 20.9 vs 41 ± 41.5 CFU-E, $p = 0.01$; 52.6 ± 44 vs 109 ± 21.8 BFU-E, $p = 0.007$, and 2.3 ± 1.8 vs 8.7 ± 6.5 CFU-GEMM, $p = 0.02$). The growth of CFU-GM did not differ in these patients from growth in controls (on average 69.3 ± 69 vs 66.5 ± 22.7 CFU-GM; *p* NS). Morphological characteristics of colonies were similar both in patients and in control subjects. The addition of anti-TNF-α and of cytokines (IL-7, IL-2, or IL-2 plus IL-7) determined an increased growth of in vitro colonies, especially after addition of IL-2 plus IL-7 (Fig. 2).

The content of primitive progenitors was evaluated by the LTC-CFC assay (Fig. 3*A*). The number of primitive BM progenitor cells was reduced 9-fold compared with those in control subjects (1.8 ± 1.4 vs 15.4 ± 3.6 LTC-CFC/10^6 BMMCs; $p = 0.0004$). The weekly addition of anti-TNF-α or the cytokines IL-7 or IL-2 to BMMC LTC in three CVID patients determined a considerable increase in the cellularity, in parallel with a significant increased number of secondary CFCs (Fig. 3*B*).

Measurements of spontaneous cytokine production in BMMCs

In vitro production of IL-2 and TNF-α by short-term culture of BMMCs was determined in CVID patients (Fig. 4*A*) and compared with healthy subjects. The amount of IL-2 produced was lower in patients vs healthy controls (16.9 ± 17 pg/ml vs 50.5 ± 7.4 pg/ml; $p = 0.0009$). On the contrary, the spontaneous production of TNF-α was significantly greater in patients (78.2 ± 87.4 pg/ml vs 10.4 ± 6.4 pg/ml; $p = 0.0007$). Fig. 4*B* shows the kinetic study of IL-2 and TNF-α production at 24, 48, 72, and 96 h in CVID patients and controls. In CVID patients, IL-2 production remained low with respect to the controls, whereas TNF-α production progressively increased.

Immunohistochemistry of BM stromal cells and IL-7 production

Next, we generated stromal cell layers from BMMCs of patients and controls. The stromal layers cultured on chamber slides were positive for CD68, vimentin, and CD14 but negative for S100 and CD34 molecules, indicating that these were preferentially of the macrophage/monocyte lineage origin. Upon light microscopy examination, the majority (75%) of these cells appeared as moderately large cells, frequently rounded, with abundant cytoplasm. In control subjects, ~90% of the stromal cells exhibited a different morphology characterized by irregular or spindle shape and branching cytoplasmic processes (fibroblast-like) as shown in Fig. 5.

Compared with healthy subjects, stromal cells from patients spontaneously produced lower levels of IL-7 (0.8 ± 0.1 pg/ml vs 0.3 ± 0.1 pg/ml, respectively; $p = 0.02$; Fig. 6).

Discussion

Regulation of hemopoiesis and maintenance of homeostasis in BM require a well balanced interaction between the hemopoietic cells and the immune system. It has been shown that immune deregulation in autoimmune and chronic inflammatory disorders may

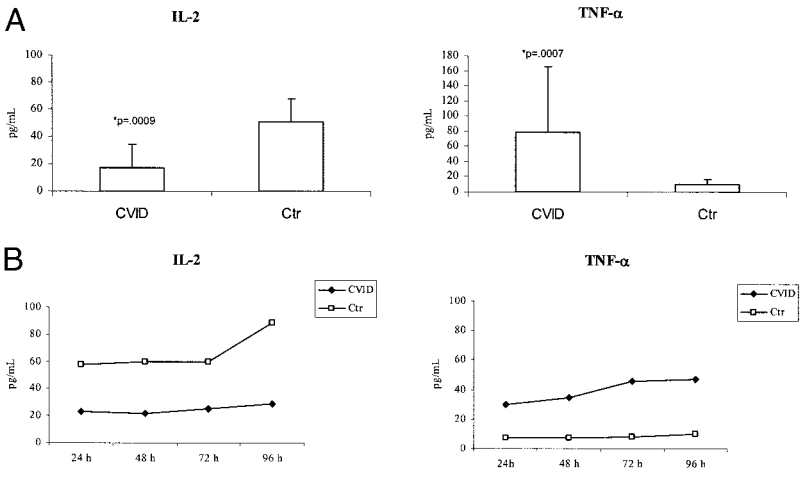

FIGURE 4. Cytokine production from BMMCs in patients with CVID. *A,* BMMCs (1×10^6 cells) were cultured in 1 ml of RPMI 1640 for 24 h. Cell supernatants were obtained from CVID patients and eight healthy controls (Ctr). Supernatants were assayed for the presence of cytokines by ELISA (see *Materials and Methods*). *B,* BMMCs (1×10^6/well), obtained from three CVID patients and two controls, were cultured, and the supernatants were collected after 24, 48, 72, and 96 h for IL-2 or TNF-α kinetic study by ELISA.

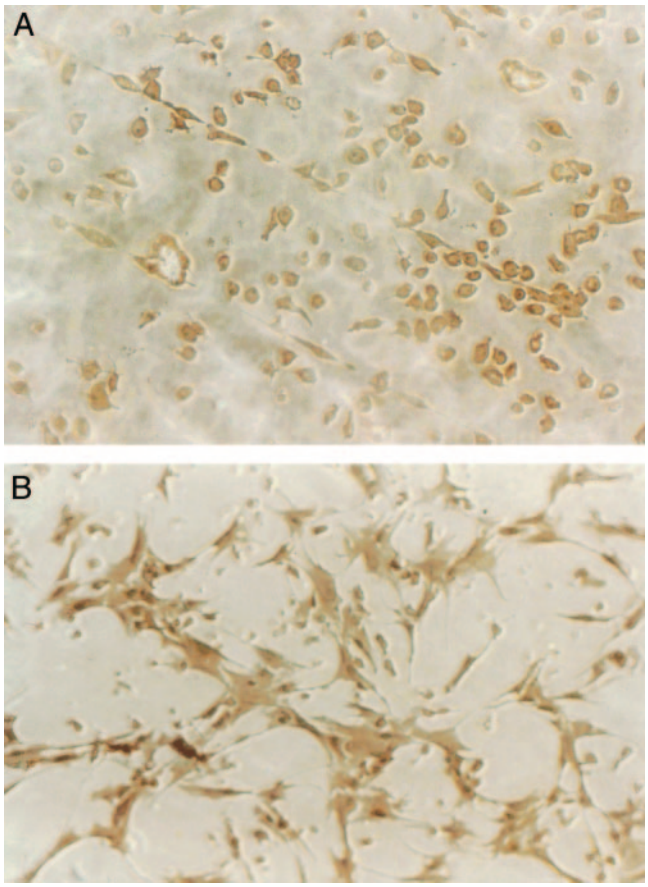

FIGURE 5. Immunohistochemical staining with anti-CD68 of BM stromal cells from patients (*A*) and healthy controls (*B*). The stromal cells were sorted onto slides and fixed in acetone-ethanol (50:50) before staining with anti-CD68, as described in *Materials and Methods.* The brown color denotes the presence of CD68 Ag. The slides were analyzed by light microscopy (×40).

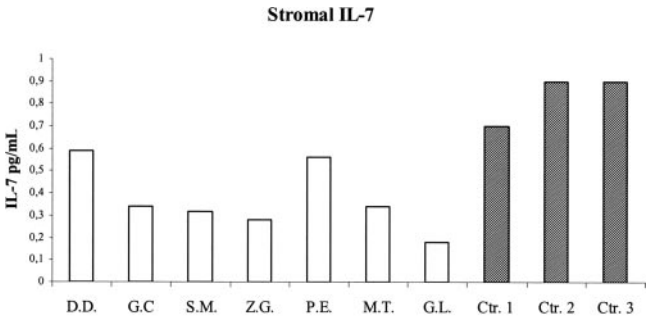

FIGURE 6. Stromal IL-7 production in patients (initials on *x*-axis) and healthy controls (Ctr). BMMCs were cultured until stromal confluence; then the cells were collected by trypsinization and cultured at a concentration of 1×10^6 cells/ml. Supernatants obtained from seven CVID patients (□) and three healthy subjects (■) were collected after 24 h of culture and measured for IL-7 production by ELISA.

modulate the function of BM hemopoietic progenitor cells and/or their microenvironment, either by inflammatory cytokine production or by cell to cell interactions (23). Occurrence of cytopenias may be observed in primary immunodeficiencies, and it is particularly frequent in CVID patients (5). Defects in T cell regulation and cytokine production, abnormal apoptosis, or abnormal secretion of Igs with autoimmune features are all potential mechanisms of cytopenias in association with congenital immunodeficiency (6–11). In addition, altered hemopoiesis may be associated with a variety of functional and immunophenotypic abnormalities at BM level, due to augmented local production of inflammatory cytokines, increased T cell activation, or intrinsic hemopoietic and stromal cell abnormalities.

In view of the current interest in exploring the role of hemopoietic stem cell transplantation, we evaluated BM progenitor cell reserve and function and stromal cells in 11 CVID individuals. In addition, we analyzed the cytokine production of BMMCs and the morphology of stromal cells established with long term cultures of BMMCs. The cytokines were selected on the basis of available data, showing the role of such molecules in impaired hemopoiesis in humans as well as for their regulatory effects on human hemopoietic progenitor cells (24, 25).

A significant quantitative and functional defect in hemopoietic progenitor cells was observed in our patients, as indicated by an altered clonogenic potential of BM-committed progenitor cells and a substantial reduction in primitive progenitors (LTC-CFC). These abnormalities may represent either an intrinsic progenitor cell defect or a secondary progenitor cell damage in response to an underlying inflammatory process within the BM microenvironment. In this regard, the reduced erythroid compartment and the tendency to an expansion of CFU-GM may be related to the frequent recurrences of infectious episodes observed in CVID patients. Compared with healthy controls, patients displayed an altered cytokine production by BMMCs, characterized by decreased levels of IL-2 and increased levels of TNF-α.

Although the proportion of total $CD34^+$ cells was within the range of adult controls, phenotypically primitive $CD34^+CD38^-DR^+$ cells, which include lymphoid precursor cells, were decreased.

With regard to B cell development, we established a significant correlation between the frequency of $CD34^+CD19^+CD10^+$ (pro-B cells), the $CD34^-10^+$ (pre-B cells) in the BM, and the number of peripheral blood $CD19^+$ cells. This finding, together with a decrease in the proportion of BM pre-B cells, indicates the involvement of BM in T and B cell differentiation, at least in selected CVID patients. These findings correlated with a significant decrease in total lymphocyte counts and depletion of $CD4^+$ T cells expressing predominantly the $CD45RA^+CD62L^+$ phenotype. Also, the $CD4^+CD45RA^+CD31^+$ T cell subset was significantly reduced in our cohort and correlated with the diminished content of TRECs previously observed (18), suggesting thymus involvement in these patients. Furthermore, we cannot rule out a defective egress of naive T lymphocytes from the thymus, which is regulated by sphingosine 1-phosphate receptor 1 (26). Indeed, it is possible that the T cell defect in CVID may occur at multiple levels, including egress from thymus.

IL-2 is a cytokine that increases proliferation and function of $CD4^+$ and $CD8^+$ T lymphocytes, B lymphocytes, and NK cells in vitro. Several studies have also shown positive effects of IL-2 on apoptosis; the addition of IL-2 to the lymphocyte cultures reduces apoptosis of PBMCs obtained from patients with secondary immunodeficiency (27). Recently, IL-2 was administered for a long period to CVID patients with associated numerous T cell defects, demonstrating a partial correction of cell-mediated immunity as well as of Ab production after immunization (28). In contrast, the suppressive effect of TNF-α on marrow progenitor cells has long been demonstrated and is mediated by affecting cell viability or by modulating the expression of numerous cytokine receptors on the cell surface (29). TNF-α is a well-known negative regulator of

hemopoiesis that induces functional FAS on $CD34^+$ cells (30, 31) and promotes their differentiation (32). The elevated TNF-α levels and the decreased IL-2 production observed in our patients might be involved in mediating the apoptotic depletion of progenitor cells as demonstrated by the altered growth of in vitro colonies observed, also in the more primitive compartment. In fact, the addition of anti-TNF-α Ab and of the cytokines IL-7 and IL-2, alone or in combination, to CFC and LTBMC assays determined an increased growth of in vitro colonies, especially with IL-2 plus IL-7, suggesting an important role of these factors for committed and uncommitted progenitor cells.

In vivo and in vitro, hemopoiesis occurs in association with the complex network of cell types found in the stroma including nonhemopoietic (fibroblasts, adipocytes, and endothelial cells) and hemopoietic cells (macrophages and T cells) (19). Progenitor cell growth and differentiation depends on their interaction with stromal cells. Low levels of cytokine production can be more effective when local concentration is increased by cell-cell contacts and by the binding of cytokines to the extracellular matrix (20, 33). The prevalence of macrophage-like cells in long term bone marrow culture, rather than typical "fibroblast-like" cells, suggests an altered composition of the BM stroma, possibly linked to an underlying inflammatory process within the BM microenvironment.

A central function of stromal cells is the IL-7 production (34). Recent evidence shows that IL-7 acts as a master regulator of T cell homeostasis, expanding both the naive and memory T cell populations (35, 36). IL-7 primarily acts as a growth and antiapoptotic factor for B and T cell precursors, and its production is a critical step for the beginning of B and T lymphopoiesis starting from stem cells. Moreover, IL-7 induced and required the antiapoptotic protein MCL-1 to mediate lymphocyte survival. Thus, MCL-1, which selectively inhibits the proapoptotic protein BIM, is essential both early in lymphoid development and later on in the maintenance of mature lymphocytes (37). Compared with controls, an altered stromal cytokine production was observed in CVID patients, characterized by decreased IL-7 levels. This pattern suggests a critical role of BM accessory cells in the altered cytokine secretion in CVID patients and raises the possibility that diminished availability of IL-7 might contribute to accelerated death for apoptosis of stem cell precursors and to the development of $CD4^+$ T cells depletion in CVID patients.

In conclusion, a reduced content of in vitro committed and primitive progenitors was observed in BMMCs of CVID patients, associated with phenotypic abnormalities, and an altered stromal cell composition and cytokine production. These abnormalities may be due, at least in part, to the increased TNF-α production by inflammatory cells in the BM and to the decreased IL-2 levels. We hypothesize that the damage of T cell compartment may be at least partially due to an altered generation of new T cells starting from the hemopoietic stem/progenitor cells. Based on these results, therapeutic administration of some cytokines (i.e., IL-2 plus IL-7) and the use of antiapoptotic factors as a future strategy may be useful for the treatment of some patients affected by CVID syndrome.

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Disclosures

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