

# The Circulating Dendritic Cell Compartment in Patients with Chronic Lymphocytic Leukemia Is Severely Defective and Unable to Stimulate an Effective T-Cell Response<sup>1</sup>

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## ABSTRACT

Chronic lymphocytic leukemia (CLL), the most frequent leukemia in the Western world, is characterized by a profound dysregulation of the host immune system that has a marked impact on the clinical course of the disease. To date, the competence of the circulating dendritic cell (DC) compartment in CLL patients has not been investigated. To address this issue, we sorted DC precursors from the peripheral blood of CLL patients and found a profoundly altered compartment as compared with normal donors. CLL DCs proved a morphologically and phenotypically immature population, lacking the maturation antigen CD83 and the costimulatory molecule CD80, unable to induce a significant proliferative response in allo-mixed lymphocyte reaction, with a reduced ability to release interleukin 12 and to drive a type 1 T-cell response. To investigate whether these defects could be ascribed to inhibiting soluble factors released by the leukemic clone, DCs were generated *in vitro* from normal monocytes in the presence of allogeneic CLL cells. The addition of CLL cells induced similar markers of abnormal maturation and functional impairment with an inhibition in the expression of costimulatory molecules and a reduction of their allo-stimulatory ability. The blocking of interleukin 6 activity was able to revert the inhibition in a proportion of patients. Taken together, these findings indicate that mechanisms of tumor-induced DC inhibition are operational in CLL patients, resulting in both maturative and functional defects in the circulating DC compartment, with a potential functional impact in the regulation of *in vivo* T-cell immune responses.

## INTRODUCTION

DCs<sup>3</sup> are professional APCs that display a unique capacity to stimulate naïve T cells and initiate primary immune responses (1–3). DCs also play a critical role in the induction of peripheral immunological tolerance, driving the differentiation of naïve T cells into distinct classes of effectors and regulatory cells or, in special circumstances, their deletion (4–6). Distinct DC subsets, of both myeloid and lymphoid origin, have been identified. Recent studies suggest that several phenotypically and functionally distinct subpopulations of circulating DCs, with potentially different constitution, function, and lineage of origin, can be detected in human blood (7). Through a differential expression of peptide-MHC complexes, variable levels of costimulatory molecules and release of immune cytokines, immature and mature DCs of different lineages differ in their ability to induce T-cell tolerance or activation. In particular, immature DCs of both

myeloid and lymphoid origin, and DCs differentiated from lymphoid precursors, have been described as inducers of a prevalent Th2 response (8, 9). Thus, changes in the DC compartment composition and properties may differentially regulate *in vivo* cell-mediated immunity.

In solid tumors, a functional impairment of DCs appears as one of the mechanisms of tumor escape from the control of the immune system. Several studies have reported that DCs isolated from tumor-bearing animals and cancer patients show a number of phenotypic and functional abnormalities (10–12). Tumor supernatants are able to inhibit the full maturation of functional DCs, and several tumor-secreted factors have been indicated as responsible for these effects, including VEGF, monocyte-colony stimulating factor, and IL-6 (13, 14). Because functionally competent DCs can be generated *in vitro* from PB progenitors of cancer patients (15–17), tumor-derived factors may inhibit the *in vivo* differentiation of DCs in cancer-bearing hosts, decreasing the number of circulating mature DCs. Data reported recently suggest that quantitative and functional abnormalities can also be present in the circulating DC compartment of patients with hematological malignancies (12, 18, 19). However, the low percentages of DC precursors in the PB, in particular in leukemic patients with a large burden of circulating tumor cells, have largely impaired a precise functional characterization of the DC compartment in these patients.

CLL is the most frequent leukemia in the Western world, accounting for ~30% of all cases of leukemia in adults (20). The clinical course of the disease is predominantly determined by a profound dysregulation of the immune system, to an extent that it has been estimated that ~60% of deaths are caused by bacterial or viral infections (21, 22). Multiple abnormalities have been described within the accessory nonleukemic T-cell compartment of CLL patients, suggesting a chronic state of incomplete activation *in vivo*, with a consequent induction of an anergic state for CLL T cells (23–28). Moreover, functional defects have also been reported in the NK population (29–31). Despite the abundance of information on T and cytotoxic cell defects in CLL patients, very few data are currently available on the status of the DC population in this disease and on its regulatory role on T-cell responses.

In this study, we investigated the status and function of the circulating DC compartment, and its potential regulatory role on T-cell responses, in CLL patients with active disease. For this purpose, we used enriched DC populations isolated directly from PB samples that included both myeloid and lymphoid subgroups of circulating DCs. Phenotypic and functional analysis of these sorted fractions was aimed at assessing whether a functional professional APC population is present in these patients. Our data show that circulating DCs in CLL patients display an altered phenotype compared with healthy subjects and differ in their capacities to direct T-cell differentiation. Furthermore, soluble factors derived from neoplastic CLL cells are capable of inhibiting the maturation of allogeneic normal monocyte-derived DCs, suggesting that mechanisms of tumor-induced DC inhibition may be operational *in vivo* in this disease and negatively impact immune responses in these patients.

Received 1/31/03; accepted 5/28/03.

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<sup>1</sup> Supported by Associazione Italiana per la Ricerca sul Cancro, Istituto Superiore di Sanità, and Ministero dell'Istruzione, Università e Ricerca, Progetto Ateneo, University “La Sapienza” of Rome.

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<sup>3</sup> The abbreviations used are: DC, dendritic cell; APC, antigen presenting cell; Th, T-helper; VEGF, vascular endothelial growth factor; IL, interleukin; CLL, chronic lymphocytic leukemia; NK, natural killer; PBMC, peripheral blood mononuclear cell; FBS, fetal bovine serum; mAb, monoclonal antibody; GM-CSF, granulocyte monocyte-colony stimulating factor; PB, peripheral blood; FACS, fluorescence-activated cell sorter; PE, phycoerythrin; cpm, counts per min; CML, chronic myeloid leukemia; MLR, mixed lymphocyte reaction.

## MATERIALS AND METHODS

**Patients and Blood Samples.** We studied patients with CLL with florid disease who had never received antileukemic therapy. White cell counts ranged from 13 to  $100 \times 10^9$ /liter (mean  $52 \times 10^9$ /liter) and CD19+/CD5+ leukemic cells accounted for 57–92% (mean 77%) of the circulating mononuclear cells. Control samples were obtained from healthy donor buffy coats. PBMNCs were separated by density gradient centrifugation (Lymphoprep; Nycomed Pharma, Oslo, Norway) and cultured in RPMI 1640 (Life Technologies, Inc., Mulgrave, Victoria, Australia) supplemented with 10% FBS (HyClone, South Logan, UT), 0.3 mg/ml L-glutamine, and 1% Pen-strepto (Euro-Clone), referred as complete medium, at 37°C in 5% CO<sub>2</sub> in air. In some cases, patient and control PBMNCs were cryopreserved in liquid nitrogen and thawed before use.

**DC Enrichment.** Circulating DC precursors were enriched from PBMNC samples using a specific immunomagnetic DC isolation kit (Miltenyi Biotec, San Francisco, CA), following the manufacturer's instructions. Briefly, pre-enriched blood DCs were obtained by depletion of T, NK, and monocytic cells by incubation with biotinylated mAbs against CD3, CD16, and CD11b, followed by addition of streptavidin magnetic beads, passage through a separating column in a strong magnetic field, and collection of the unbound fraction. In some experiments, patient samples with large leukemic infiltration were simultaneously depleted of CD19+ cells to allow a better DC recovery. Pre-enriched DCs were then stained with CD4-microbeads and the positive fraction collected as enriched DCs. Because different levels of CD4 antigen are expressed by all of the subsets of circulating DCs, this method allows the purification of the entire circulating DC compartment, including CD11c+ and CD11c- cells. A mean of  $8 \times 10^5$  cells (range, 2 to  $10 \times 10^5$ ) were obtained after processing a mean of  $110 \times 10^6$  (range, 77 to  $150 \times 10^6$ ) PBMNCs in normal donors. DCs were identified in the purified fraction as HLA-DR+ cells, negative for granulocyte, monocyte, NK cell, and T- and B-cell markers (Lin-). HLA-DR+/Lin- DCs represented on average 57% (range, 32–87%) of the purified population. In CLL, a mean of  $5 \times 10^5$  cells (range, 2 to  $8 \times 10^5$ ) were obtained from a mean of  $160 \times 10^6$  (range, 108 to  $220 \times 10^6$ ) PBMNCs, with a mean purity of the isolated fraction of 40% (range, 27–64%). We did not attempt an additional purification of the DC population from the enriched fraction to preserve a sufficient number of cells for phenotypic and functional analysis. However, experiments were designed to minimize the influence of different DC percentages in different samples on experimental results. Contaminant cells in enriched DC preparations were mainly CD4+ T cells and monocytes. The contribution of these populations to T-cell proliferation induced by DCs is assumed to be marginal. In all of the cases, isolated cells were cultured in complete medium for 24 or 48 h and then analyzed.

**Generation of DCs from Monocytes.** PBMNCs from healthy donors, isolated as described above, were plated ( $1 \times 10^7/3$  ml/well) into six-well plates in complete medium. After 2 h of incubation at 37°C, nonadherent cells were removed by gentle washing, and the adherent cells were cultured in complete medium supplemented with 100 ng/ml of IL-4 (R&D System, Abingdon, United Kingdom) and 100 ng/ml of GM-CSF (R&D). DCs were harvested for phenotypic or functional analysis after 7 days of culture.

The effects of CLL neoplastic cells on normal DC maturation were examined by generating DCs from healthy donor monocytes in Transwell six-well plates (Transwell-Clear; Costar, Cambridge, MA). The adherent fraction was obtained from  $5 \times 10^6$  PBMNCs in the lower compartment and cultured for 7 days in complete medium supplemented with IL-4 and GM-CSF as described above. Five  $\times 10^6$  PBMNCs from CLL patients or control healthy donors were added in the Transwell upper compartment at the beginning of the culture. PB samples from CLL cases used in these series of experiments contained at least 80% CD19+ cells. In inhibition experiments, Transwell cultures were supplemented with 10  $\mu$ g/ml of neutralizing antibodies against IL-6, IL-10, and VEGF. All of the mAbs were purchased from R&D.

To test the allostimulatory activity of monocyte-derived DCs, CD1a+ cells were sorted using immunomagnetic beads (Miltenyi Biotec) and used as stimulators in MLR assays.

**Cell Surface FACS Analysis.** Freshly isolated or cultured DCs were analyzed by fluorescence multicolor flow cytometry. Cells were stained for 30 min at 4°C using Ca<sup>2+</sup>/Mg<sup>2+</sup>-free PBS with 1% FBS as a diluent/wash buffer. FITC-, PE-, and PerCP-conjugated mAbs were used for triple-color staining. Nonspecific binding was measured using isotype-matched mAbs of irrelevant specificity. The antibodies used were purchased from Becton Dickinson

(Mountain View, CA) for isotype controls, PE-CD14, FITC-HLA-DR, PerCP-HLA-DR, PE-CD11c, FITC-lin 1 (mixture of CD3, CD14, CD16, CD19, CD20, and CD56); PharMingen (San Diego, CA) for FITC- and PE-CD1a, FITC- and PE-CD83, FITC-CD80, PE-CD86, and FITC-CD40; and Dako (Glostrup, Denmark) for PE-HLA-ABC. Data acquisition was performed on a FACScan flow cytometer (FACScan; Becton Dickinson). Ten-thousand events were evaluated for each sample (5000 in the case of sorted DCs). MFI and the relative cell frequency expressing respective surface markers were analyzed using the CellQuest software program (Becton Dickinson).

**MLR Assay.** From 5 to  $20 \times 10^3$  irradiated (30 Gy) DCs were used as stimulator cells for allogeneic PBMNCs obtained from healthy donors. Stimulators were added to the PBMNCs ( $10^5$  cells/well) in 96-well round-bottomed microtest culture plates. After 5 days of incubation, cells were pulsed with 1  $\mu$ Ci of [<sup>3</sup>H]thymidine/well for the last 18 h, harvested and counted. Tests were performed in triplicate, and results were expressed as the mean cpm. The levels of [<sup>3</sup>H]thymidine uptake by stimulator cells alone were always <100 cpm.

**T-Cell Polarization Assay.** DCs isolated from healthy donors and CLL patients were incubated *in vitro* in complete medium for 24 h and then cocultured with allogeneic normal PBMNCs. Cocultures were performed in round-bottomed 96-well plates. Responding T cells were plated at  $1 \times 10^5$  cells/well with  $1 \times 10^4$  stimulator DCs/well. After 6 days, cells were harvested and analyzed for cytokine production by intracellular immunofluorescent staining.

**Intracellular Cytokine Staining.** Cells were analyzed for cytokine production using the Fastimmune system (Becton Dickinson), following the manufacturer's instructions. Briefly, cultured cells were harvested, washed, resuspended in complete medium added with Brefeldine A (Sigma Aldrich) 10  $\mu$ g/ml to inhibit intracellular transports, and incubated for 4 h at 37°C in 5% CO<sub>2</sub> in air. When indicated, phorbol 12-myristate 13-acetate 25 ng/ml and ionomycin 1  $\mu$ g/ml were added for activation of T cells. Cells were then washed and stained with cell surface-conjugated mAbs as described. For intracellular staining, cells were then incubated for 10 min in FACS Lysing Solution (Becton Dickinson), washed, resuspended in FACS Permeabilizing Solution (Becton Dickinson) for 10 min, stained with anticytokine conjugated mAbs, and analyzed on a FACScan flow cytometer as described. Antihuman IL-10, IL-12, and IFN- $\gamma$  mAbs were purchased from PharMingen.

**ELISA Assays.** PBMNCs from CLL patients were cultured in complete medium at  $1 \times 10^6$  cell/ml. After 48 h, supernatants were harvested and tested for cytokine production using commercial quantitative sandwich immunoassay kits from R&D System (Quantikine for human IL-6, IL-10 and VEGF). Quantikine kits for human IL-10 and IL-12 were also used to test cytokine production by isolated DCs.

**Cytomorphological Analysis.** Cytospins were prepared by centrifuging  $1 \times 10^4$  cells in 200  $\mu$ l medium on to slides at 400 rpm for 5 min. Slides were then dried and stained by May-Grunwald-Giemsa. Stained slides were used for cell lineage and morphological evaluation.

**Statistical Analysis.** The statistical significance between independent groups was determined using Student's *t* test for matched pairs.

## RESULTS

**Phenotype of Circulating DCs Isolated from Normal Donors and CLL Patients.** Circulating DCs were enriched by immunomagnetic separation from the PB of 10 healthy subjects to allow their phenotypic and functional analysis *in vitro*. They showed light scatter properties of immature cells and lacked typical DC markers (Fig. 1A). After overnight *in vitro* culture in the presence of FBS, normal DCs acquired a typical DC morphology (Fig. 1B) and became positive for most specific DC surface markers, including CD1a, CD83, CD80, CD86, CD11c, CD40, and high levels of HLA-class I and class II (Table 1), although the levels of these antigens varied between donors. It is worth noting that only a small percentage of CD1a+/CD83+ double-positive cells was observed: two DC populations, one CD1a+/CD83-, and one more mature CD1a-/CD83+ were present in the sorted fraction.

Ten different CLL samples from untreated patients were also analyzed. Freshly isolated immature CLL-DCs did not show marked

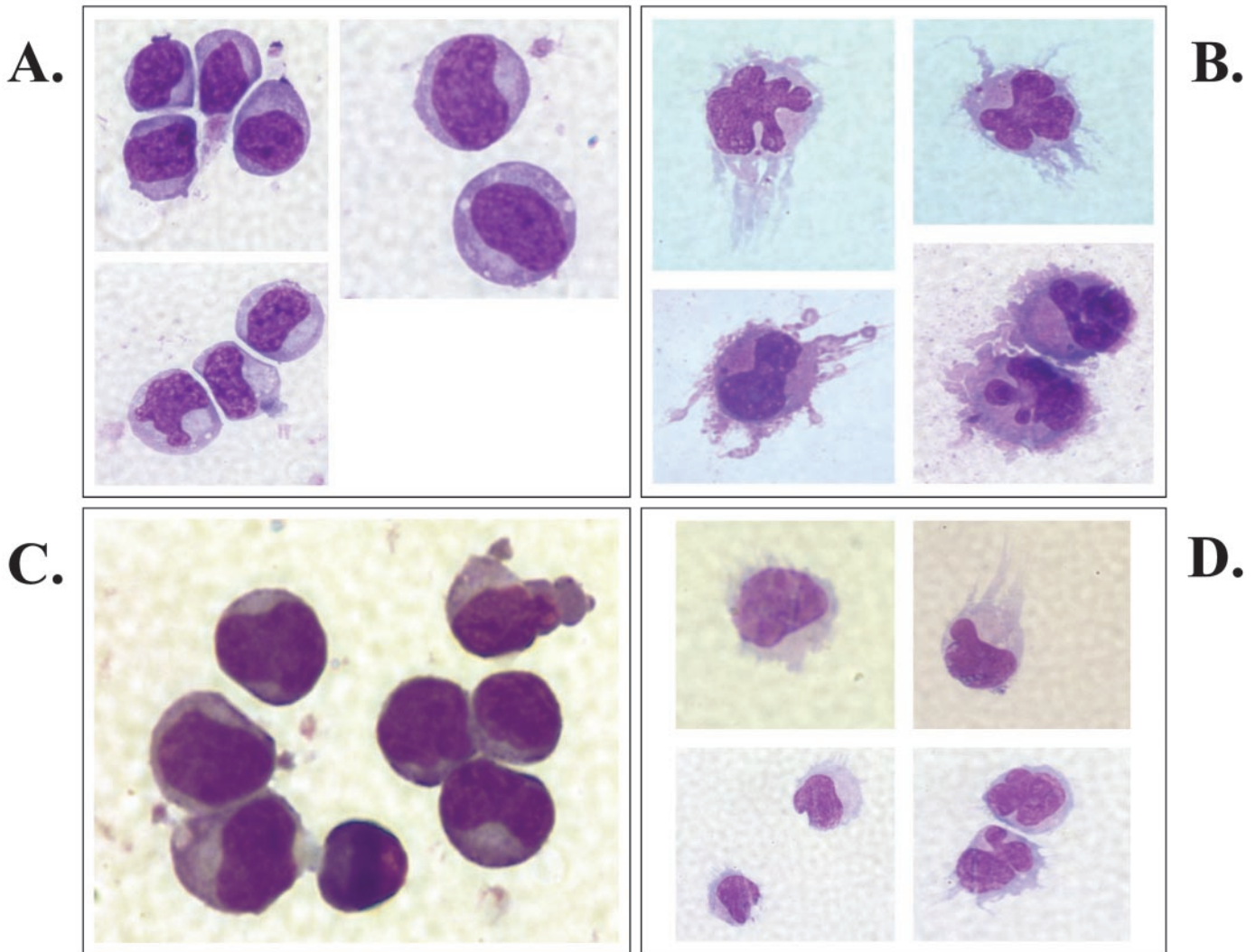


Fig. 1. Morphology of sorted DCs. DCs sorted from the PB of healthy donors (A and B) and CLL patients (C and D) just after sorting (left panels) and after 24 h of *in vitro* culture (right panels). Slides were stained with May-Grünwald-Giemsa, original magnification  $\times 100$ .

morphological and phenotypic differences compared with the normal counterparts (Fig. 1C). However, the morphological and phenotypic maturation induced by the *in vitro* culture period in normal immature DCs could be only partially detected in CLL-DCs. Although they changed into bigger and more granular cells, the typical DC morphology with large and distributed membrane processes was not observed. Instead, CLL-DCs for the most part displayed a polarized distribution of dendrites, similar to what observed in plasmocytoid DCs (Fig. 1D). Moreover, the fine dispersion of the nuclear chromatin in CLL-DCs and the presence of nucleoli, not observed in normal DCs, showed their incomplete degree of maturation. CLL-DCs acquired the surface marker CD1a specific for DCs, coupled with the expression of the costimulatory molecule CD86, with variable levels of CD11c and

CD40. They also expressed HLA-class I and HLA-class II surface antigens, although at a lower level of intensity than normal DCs. Notably, purified DCs from CLL patients lacked the maturation marker CD83 in all of the cases but two (CLL1 and CLL7 DCs both expressed CD83 on 25% of the cells) and did not express the co-stimulatory molecule CD80. Table 1 shows the expression of surface antigens in the enriched population from CLL samples. The addition of tumor necrosis factor  $\alpha$  for 72 h to CLL-DCs in culture did not induce the appearance of additional signs of maturation (data not shown).

**Functional Properties of Circulating DCs Isolated from Normal Donors and CLL Patients.** We then sought to investigate the functional properties of circulating DCs isolated *in vitro*. The most char-

Table 1 Surface markers of enriched DC populations from 10 healthy donors and 10 CLL patients

| % Positive cells | Lin <sup>-</sup> /DR <sup>+</sup> | CD1a        | CD83        | CD80        | CD86        | CD11c       | CD40        | HLA-DR<br>(mean fluorescence intensity) |
|------------------|-----------------------------------|-------------|-------------|-------------|-------------|-------------|-------------|---|
| Healthy donors   |                                   |             |             |             |             |             |             |   |
| Mean $\pm$ SD    | 57 $\pm$ 18                       | 22 $\pm$ 18 | 22 $\pm$ 11 | 26 $\pm$ 18 | 44 $\pm$ 19 | 29 $\pm$ 18 | 33 $\pm$ 22 | 3390 $\pm$ 1113                         |
| Range            | 32–87                             | 5–60        | 7–43        | 7–70        | 18–80       | 11–55       | 6–72        | 1657–5170                               |
| CLL patients     |                                   |             |             |             |             |             |             |   |
| Mean $\pm$ SD    | 40 $\pm$ 11                       | 26 $\pm$ 8  | 5 $\pm$ 11  | 0.5 $\pm$ 2 | 28 $\pm$ 11 | 16 $\pm$ 20 | 18 $\pm$ 16 | 753 $\pm$ 342                           |
| Range            | 27–64                             | 14–38       | 0–25        | 0–5         | 13–42       | 6–70        | 8–48        | 317–1195                                |



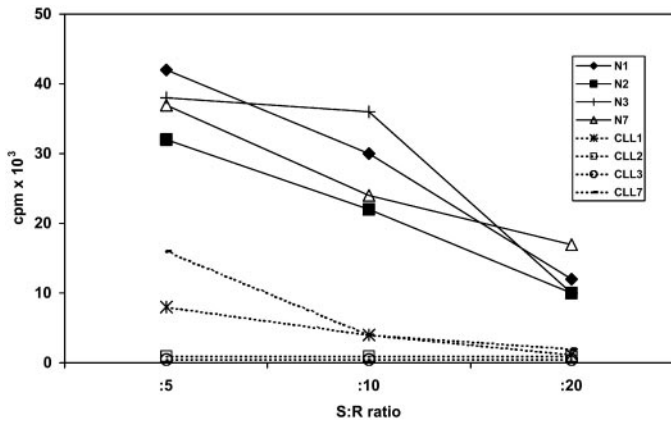


Fig. 2. Allostimulatory ability of sorted DCs. DC populations enriched from healthy donors and CLL patients were irradiated and cultured with allogeneic PBMCs at different stimulator:responder ratio. [ $^3\text{H}$ ]Thymidine incorporation was measured after 5 days. Allogeneic MLR were performed with the same source of responder T cells for each experiment, involving one couple normal/CLL DCs. The allostimulatory activity of normal DCs was elevated and that of CLL DCs absent or very low. Results from 4 normal donors and 4 CLL patients are presented in the figure. The assays were performed in triplicate and the mean values are shown. Triplicates were always consistent. *S:R ratio*, stimulator:responder ratio.

acteristic functional feature that discriminates DCs from other APCs is their ability to induce proliferative T-cell responses, as determined by allostimulatory assays. Isolated normal DCs, after *in vitro* culture, demonstrated their functional maturity exhibiting a good allostimulatory capacity in MLR assay. In contrast, little, if any, allostimulatory effect was shown by CLL-DCs, even at high stimulator:responder ratios (Fig. 2). The low or absent allostimulatory capacity of CLL samples is unlikely to be because of the lower number of DCs in the enriched fraction compared with normal samples, because even a 2-fold compensation still failed to render the activity similar. These findings were consistent with the phenotypic data (CLL1 and CLL7 were the only patients to show some degree of activity, see above) and confirmed the functional impairment of CLL-DCs.

To better investigate the ability of DCs isolated from PB to influence T-cell responses, cytokine production was explored by intracellular staining and ELISA analysis in normal and CLL samples. We tested the production of IL-10 and IL-12 in isolated DC samples. ELISA assays on supernatants of DC cultures showed a basal production of low doses of both cytokines by enriched DC populations from normal donors and CLL patients (mean IL-10 and IL-12 concentration: 147 pg/ml and 33 pg/ml, respectively). Given the difference in DC composition of these populations, IL-10 and IL-12 production by normal and CLL-DCs was compared by intracellular staining considering the IL-12:IL-10 ratio (percentages of IL-12-producing cells:percentages of IL-10-producing cells). In fact, we knew DCs to be the only source of these cytokines in the enriched population (IL-10 and IL-12 production was zero in the negative non-DC fraction). Normal DCs cultivated *in vitro* for 48 h were found to spontaneously produce in almost equal percentages IL-10 and IL-12 in five different samples tested (Fig. 3; Table 2). In three of the four cases analyzed, CLL-DCs showed a lower ability to produce IL-12; the percentage of IL-12-positive cells was found to be at the most one half of the cells positive for IL-10 production. The fourth CLL patient studied showed equal percentages of IL-10 and IL-12 producing cells. Interestingly, this patient was one of the two whose isolated DCs showed CD83 expression at the phenotypic analysis (CLL1).

To assess the ability of different DCs to induce a type 1 *versus* a type 2 T-cell polarization, allogeneic PBMCs were cultured with enriched DC fractions from normal donors and CLL patients, and

cytokine production by T cells was investigated by intracellular cytokine staining. Priming by normal DCs induced IFN- $\gamma$  production by CD3+ T cells but also IL-10 production, whereas the same T cells primed by CLL-DCs produced only IL-10 (Fig. 4). Reproducible results were obtained in three independent experiments.

Thus, DCs isolated from normal donors were able to produce IL-12 and to induce allogeneic T cells to differentiate into IFN- $\gamma$ -producing effector T cells. In contrast, DCs isolated from CLL patients showed a reduced ability to release IL-12 and could only induce the differentiation of IL-10-producing type 2 cells.

**Inhibitory Effects of CLL PBMCs on Normal Monocyte-derived DC Development and Function.** To investigate whether the above described peculiarities of circulating CLL-DCs could be ascribed to inhibitory soluble factors released by the leukemic clone, we next designed a series of experiments in which DCs were generated *in vitro* from normal monocytes using GM-CSF and IL-4. Cultures were carried out in Transwell plates, and PBMCs from CLL patients were added in the upper compartment at day 0. All of the CLL PB samples used for these experiments comprised >80% of CD19+ B cells.

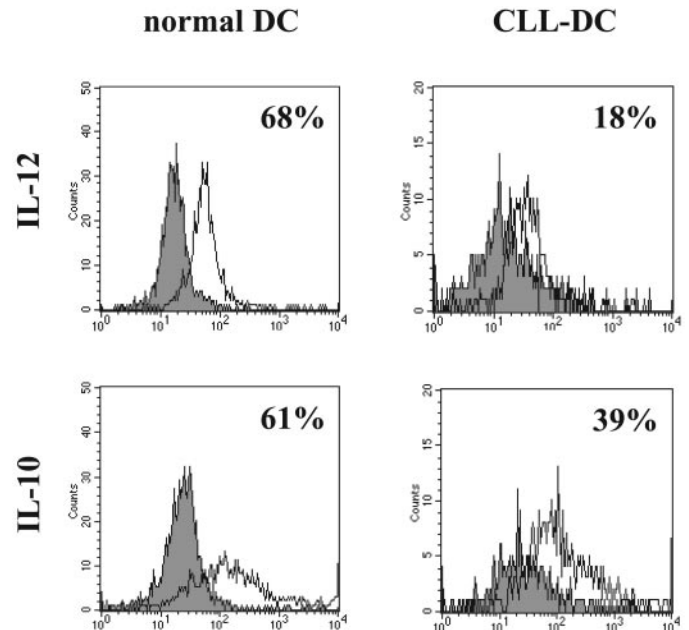


Fig. 3. Cytokine production by sorted DCs. Enriched DC populations from healthy donors, cultivated *in vitro* for 48 h and analyzed by intracellular cytokine staining, were found to spontaneously produce almost equal percentages of IL-10 and IL-12 (results representative of four independent experiments). DCs from CLL patients showed a reduced ability to secrete IL-12 in three of four cases analyzed (one representative case is illustrated). The fourth patient (not shown) displayed a normal pattern of cytokine production. Shaded histograms, isotype-matched negative controls. The percentage values showed in the figure are calculated on the basis of the number of events with increased fluorescence as compared with negative control.

Table 2 Percentage of IL-10- and IL-12-producing cells in enriched DC populations from normal and CLL samples

| Sample | % Producing cells |       |             |
|--------|-------------------|-------|-------------|
|        | IL-12             | IL-10 | IL-12/IL-10 |
| N1     | 37                | 39    | 0.94        |
| N2     | 52                | 60    | 0.86        |
| N3     | 68                | 61    | 1.11        |
| N4     | 64                | 60    | 1.06        |
| N6     | 45                | 40    | 1.12        |
| CLL1   | 55                | 46    | 1.19        |
| CLL2   | 16                | 31    | 0.51        |
| CLL3   | 18                | 39    | 0.46        |
| CLL5   | 15                | 24    | 0.62        |

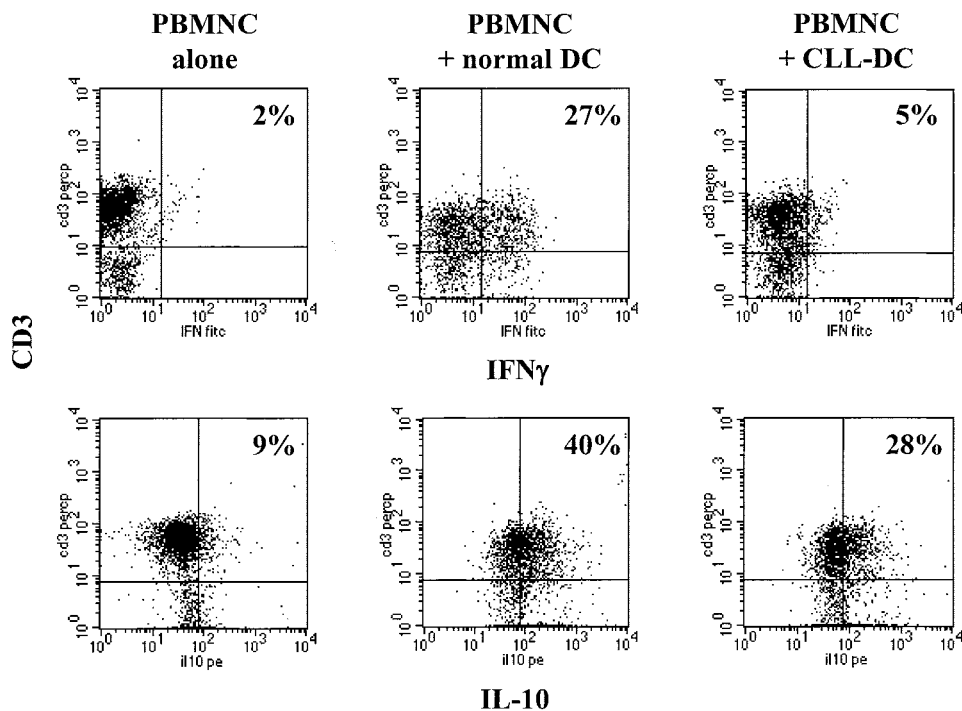


Fig. 4. Allogeneic T-cell polarization activity of sorted DCs. Allogeneic PBMNCs were cocultured with DCs populations enriched from healthy donors and CLL patients. After 5 days, T cells were stimulated with phorbol 12-myristate 13-acetate and ionomycin, and analyzed for cytokine production by intracellular staining. Whereas normal DCs induced the production of both IFN- $\gamma$  and IL-10 in the gated lymphocyte population, CLL-DCs were able to induce only IL-10 production (results representative of three independent experiments).

Control cultures were set up with normal allogeneic PBMNCs and normal purified CD19+ B lymphocytes in the upper compartment, to rule out a different effect of normal and leukemic samples linked to the different composition of the mononuclear fraction. In both cases, we did not observe any inhibitory effect (data not shown); therefore, in additional experiments, medium alone was used as a control. Normal DCs generated in the presence of CLL cells showed an altered phenotype as compared with control cultures. Variable degrees of inhibition in the expression of CD1a and CD80 were observed (Fig. 5A and Fig. 7 illustrate two examples from different experiments). The expression of the costimulatory molecule CD86, in contrast, was not affected or was even increased. These changes were statistically significant (Fig. 5B).

Functionally, the addition of CLL cells was able to modify the cytokine production profile of normal DCs. After a week in culture, normal DCs were found to synthesize both IL-10 and IL-12. In the presence of CLL cells, a reduction in the number of IL-12-producing DCs was observed, whereas the ability to produce IL-10 was increased, although not significantly (Fig. 6A; Table 3). The functional consequences of exposure to CLL-derived soluble factors were also tested in an MLR assay. CD1a+ purified DCs matured in the presence of CLL PBMNCs exhibited a reduced allostimulatory capacity, as compared with the same CD1a+ DCs cultured in the presence of medium alone (Fig. 6B).

**DC Inhibitory Factors Released by CLL PBMNCs.** CLL cells are known to release several factors potentially capable of inhibiting DC development and function. Serum levels of some of them have been found to be increased in CLL patients and could, therefore, exert their inhibitory activity *in vivo*. We sought to identify the soluble mediators of the inhibitory effect observed *in vitro* on allogeneic DC development by CLL PBMNCs using neutralizing mAbs against IL-6, IL-10, and VEGF. Indeed, literature data (32, 33) and experiments from our laboratory show increased serum levels of these cytokines in CLL patients. Blocking mAbs were added to Transwell cultures set up as described previously on day 0, and phenotypic and functional properties of cultured DCs were analyzed on day 7. In three of six experiments performed, the addition of anti-IL-6 mAbs was able to

revert the inhibitory effect of CLL cells (Fig. 7). mAbs against IL-10 were also able to partially revert the inhibition in two of six patients (one in association with IL-6), and anti-VEGF was active in one case (Fig. 8). To explain these heterogeneous results, cytokine production by PBMNCs from these CLL patients was tested *in vitro* using immunoenzymatic assays. As shown in Table 4, data on cytokine levels in the supernatants from the different PBMNC CLL samples paralleled the blocking activity of the corresponding mAb, indicating also a heterogeneous release of inhibiting factors from CLL cases.

In summary, these data suggest a role for IL-6 in the inhibition of DC development and function in CLL patients, but also a notable heterogeneity of inhibitory cytokine production by CLL PBMNCs and the potential involvement of other still unidentified factors.

## DISCUSSION

DCs play a pivotal role in the induction of T-cell immunity, including tumor-specific T-cell responses. Thus far, little information is available on their *in vivo* function in cancer patients. Although the *in vitro* generation of functional DCs from PB monocytes has been reported in many tumors, including CLL (34, 35), the relation between the monocyte-derived DC pathway and the differentiation of immature DCs from bone marrow precursors *in vivo* is not clear, and the ability to generate functional DCs *in vitro* may not reproduce the existence of a fully competent DC compartment *in vivo*. Therefore, we used a specific immunomagnetic system to directly isolate circulating DCs from the PB of CLL patients and normal donors, and investigated their maturative and functional status.

DCs are known to circulate in the PB in a percentage comprised between 0.05 and 0.1% of total PBMNCs (1). However, the paucity of DC-specific reagents and the need for alternative methods of DC identification (morphological features, lack of lineage-specific markers, and DR positivity) make the direct comparison of the phenotypic and functional properties of DC subsets reported in different studies difficult. The human blood DC compartment is likely to contain several phenotypically and functionally distinct subpopulations. The method we used, based on CD4 expression, may allow the purification

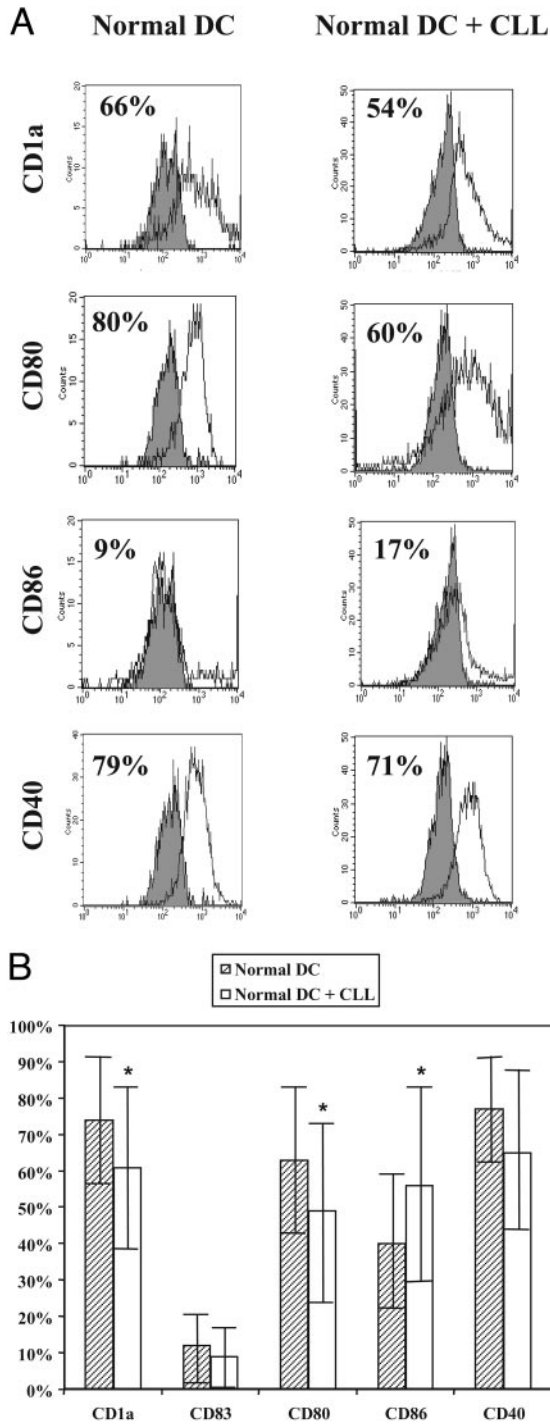


Fig. 5. Inhibitory effect of CLL cells on normal DC development. *A*, DCs were generated *in vitro* from normal monocytes, with or without the addition of CLL PBMCs, in Transwell plates. Variable degrees of inhibition of CD1a, CD80, and CD40 expression were observed in the gated large cell population when CLL cells were added. Nine independent experiments were performed with consistent results. *Shaded histograms* represent the isotype-matched negative controls. *B*, mean expression of surface antigens by normal DCs cultivated alone or with CLL cells ( $n = 9$ ). \* =  $P < 0.05$ ; bars,  $\pm$ SD.

of all of these subtypes (although data reported recently suggest the existence of a CD16+ DC subgroup that would be lost in our work; Ref. 7). In our study, isolated normal DCs showed high levels of HLA class II (DR), the presence of the costimulatory molecules CD80, CD86, and CD40, and the maturation marker CD83. Dual-color analysis of CD1a and CD83 expression allowed for the identification of two distinct subpopulations, characterized by the exclusive posi-

tivity for one of these two markers. CD1a+/CD83- cells had a smaller and less granular scatter profile, and lacked expression of CD80. By contrast, the CD1a-/CD83+ population displayed the morphological characteristics of more mature DCs, was positive for surface expression of CD80, and included a high percentage of IL-12-producing cells. By contrast, CLL-DCs, even after 2 days of culture *in vitro*, persistently lacked a mature dendritic morphology, as

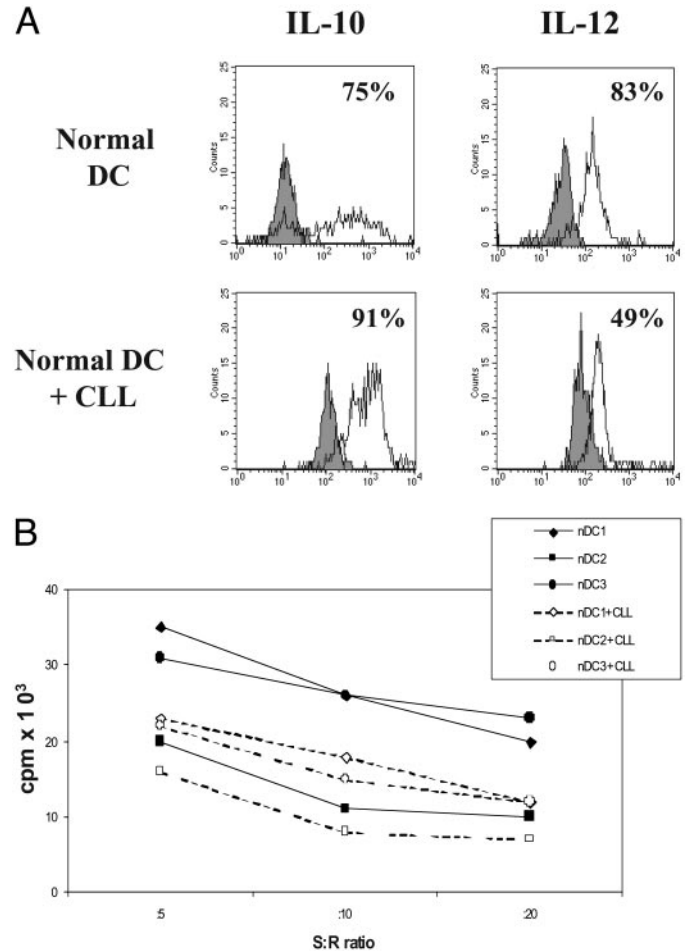


Fig. 6. Inhibitory effect of CLL cells on normal DC function. *A*, cytokine production. Addition of CLL PBMCs in Transwell plates to normal monocyte-derived DCs during their development induced the reduction of IL-12, but not IL-10, production. Results shown are gated on the DC large cell population. *Shaded histograms*, isotype-matched negative controls. *B*, allostimulatory activity. Normal monocyte-derived DCs generated in the presence of CLL PBMCs exhibited a reduced allostimulatory activity compared with controls. CD1a+ cells were sorted using immunomagnetic beads after 1 week of culture and used as stimulators. Results obtained from three different normal donor DC populations (nDC1, nDC2, and nDC3) generated with and without CLL cells from different CLL patients are shown. Differences were statistically significant in every case ( $P = 0.02, 0.01$ , and  $0.004$ , respectively). The proliferation assays were performed in triplicate, and the mean values are shown. Triplicates were always consistent. *S:R ratio*, stimulator:responder ratio.

Table 3 Percentage of IL10 and IL12 producing cells in normal DCs cultivated alone or with CLL samples

| Sample | % Producing cells  |           |       |           |
|--------|--------------------|-----------|-------|-----------|
|        | IL-12 <sup>a</sup> |           | IL-10 |           |
|        | nDC <sup>b</sup>   | nDC + CLL | nDC   | nDC + CLL |
| #1     | 82                 | 57        | 58    | 76        |
| #2     | 83                 | 49        | 75    | 91        |
| #3     | 78                 | 55        | 55    | 62        |

<sup>a</sup>  $P = 0.023$ .

<sup>b</sup> n, normal.



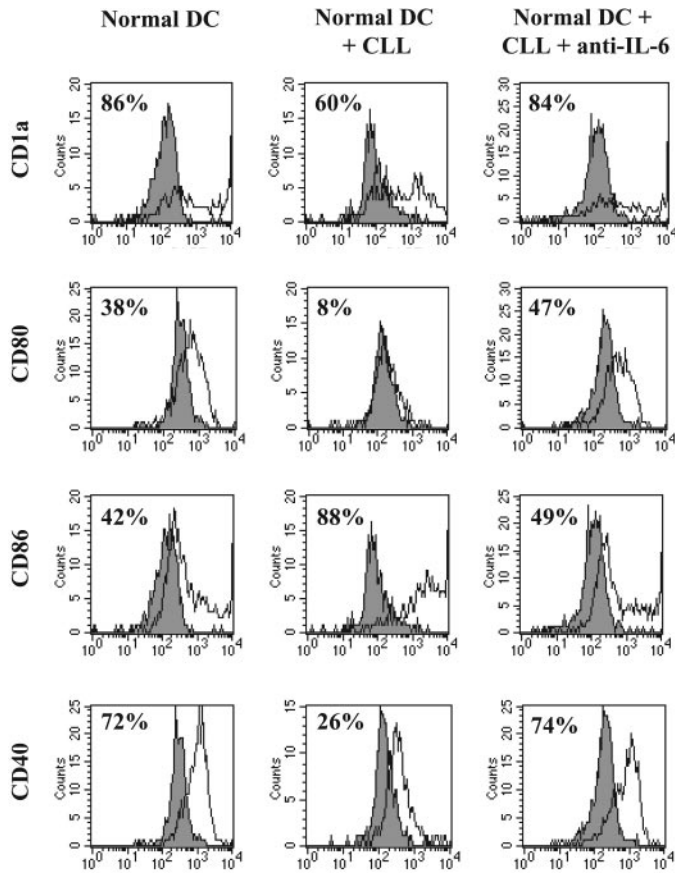


Fig. 7. Blocking IL-6 activity is capable of reverting the inhibitory action of CLL cells on normal DCs. CLL PBMCs added in Transwell plates to normal monocyte-derived DCs, as described above, induced a marked reduction of CD1a, CD80, and CD40 expression. Addition of neutralizing anti-IL-6 antibody was able to revert the defects in 3 of 6 patients tested. Similar results were obtained with anti-IL-10 in 2 patients and anti-VEGF in 1 patient (see Fig. 8). Shaded histograms represent isotype-matched negative controls.

well as CD80 and CD83 expression. These data indicate that the circulating DC population in CLL patients displays a less mature phenotype, confirmed by a reduced ability to stimulate allogeneic T-cell proliferation and to secrete IL-12. The lack of certain phenotypic markers in the CLL-DC population could be contributed by an imbalance in the composition of the entire DC compartment, with a predominance of the “lymphoid” DC population, known to express lower levels of costimulatory molecules and CD83 or, alternatively, by an altered maturation of the “myeloid” DC subgroups. On the basis of our data, it is not possible to clarify this point, although the latter hypothesis is suggested by the analysis of myeloid BDCA-1+ DCs sorted from CLL patients, carrying similar phenotypic defects (36). Whatever the case may be, the resulting lack of a normally maturing DC population with immune stimulatory activity in CLL patients might profoundly affect the ability of the DC compartment to effectively prime immune responses.

The complex alterations documented in the DC compartment of CLL patients, including the predominance of a “tolerogenic” phenotype, assume a peculiar relevance when related to the clinical characteristics of the disease. The multiple functional defects reported within the T-cell compartment of CLL patients, the anergic status of T lymphocytes, and the high incidence of infectious complications (23–28), are all phenomena that could be related to a defective DC population. Moreover, the recent acquisitions concerning the reciprocal activating interactions between DCs and NK cells (37, 38) suggest a potential negative effect of the abnormal DC compartment also on

the innate immunity in CLL patients, where defects in the ability of the cytotoxic cells to become activated, and lyse appropriate targets have been described long ago (29–31). It is worth noting that in a similar analysis performed on patients affected by CML, a disease where no specific defects of the immune system have been described,

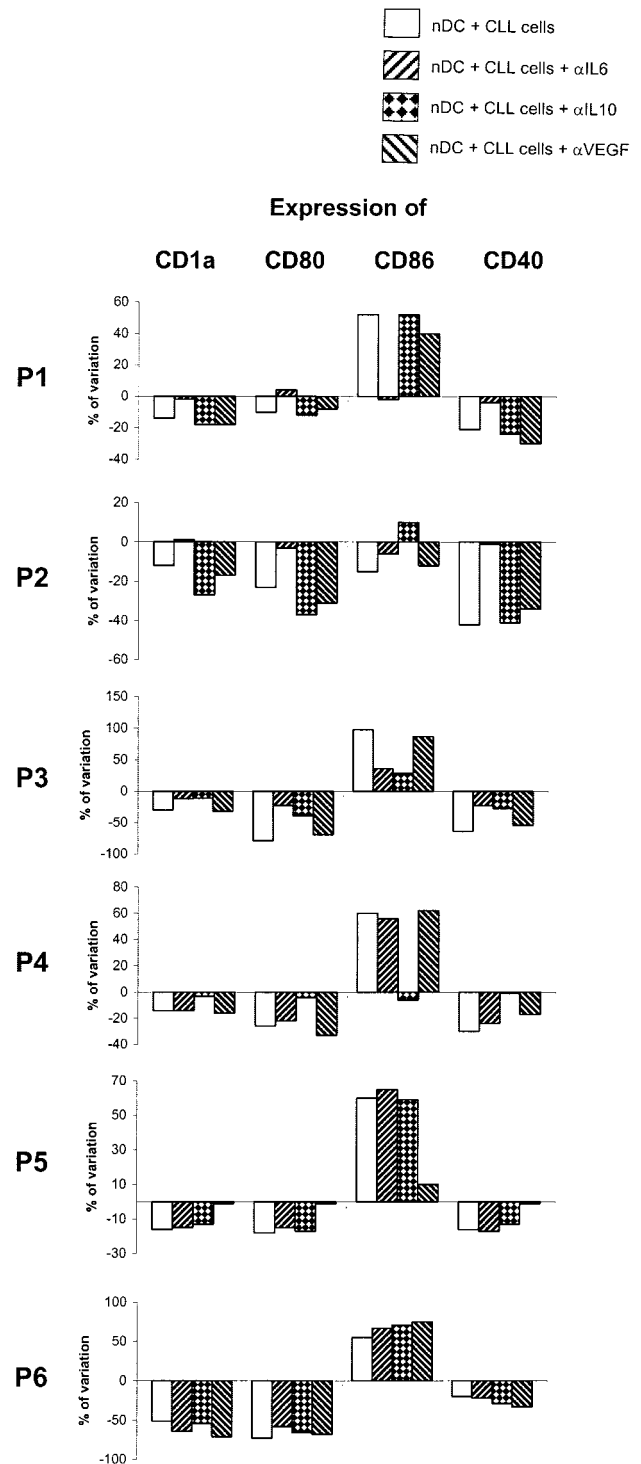


Fig. 8. Effects of blocking anticytokine mAbs on the inhibitory action of CLL cells on normal DCs. The addition of anti-IL-6, anti-IL-10, and anti-VEGF antibodies to Transwell cultures was capable of reverting the inhibitory action of CLL cells from different patients (P1–P6). The percentage of variation from the basal level (normal DCs alone) induced by the addition of CLL cells with or without blocking mAbs is shown in the figure. Anti-IL-6 was active in P1, P2, and P3; anti-IL-10 in P3 and P4; and anti-VEGF in P5. None of them was effective in P6.

Table 4 Cytokine production from CLL PBMCs samples

| Patient | Production (pg/ml) |       |      |
|---------|--------------------|-------|------|
|         | IL-6               | IL-10 | VEGF |
| P1      | 101                | 0.9   | 0    |
| P2      | 609                | 0     | 0    |
| P3      | 417                | 4.7   | 0    |
| P4      | 54                 | 10    | 0    |
| P5      | ND <sup>a</sup>    | ND    | ND   |
| P6      | 0                  | 0     | 0    |

<sup>a</sup> ND, not done, sample not available.

we observed different findings (36). Although some minor functional abnormalities could be detected, DCs isolated from CML patients appeared as a normally maturing population. In particular, CML DCs exhibited a typical myeloid morphology, elevated levels of surface costimulatory molecules, and a good allostimulatory activity. A more detailed functional analysis will assess the ability of the DC compartment to drive productive immune responses in CML patients, but the differences with what was observed in CLL are striking. These observations emphasize the potential relevance of DC functionality in determining the immune status and clinical picture of cancer patients.

In CLL, a Th2-type response may progressively dominate over a Th1-type during disease progression. In fact, an increased expression of the Th2-associated marker CD30, a reduced coexpression of CD7 by CD4+ T cells, and an enhanced production of IL-4 have been described in CLL (39, 40). DCs direct T-cell differentiation through membrane contact and cytokine release, with a particularly relevant role for IL-12, the critical Th1 polarizing cytokine. On maturation, DCs are known to produce large amounts of IL-12 as well as other cytokines, such as IL-1 $\alpha$ , IL-1 $\beta$ , IL-6, and IL-10. The ability to drive a Th1- or Th2-type response is related to the levels of IL-12 produced: lower amounts of IL-12 preferentially induce a Th2 development (6, 41, 42). We found a reduced ability to produce IL-12 in DCs isolated from CLL patients, as compared with normal controls. Furthermore, CLL PBMCs were capable of inhibiting the production of IL-12 from normal monocyte-derived DCs *in vitro*. These findings suggest that in CLL patients the defective DC compartment has a reduced ability to release IL-12, perhaps under the influence of inhibitory factors produced by the neoplastic clone. In the close cross-talk between DCs and T-cell populations, the decreased availability of IL-12 may participate to direct T-cell differentiation toward a type 2 response. This was additionally confirmed by experiments in which DCs isolated from CLL patients were cocultured *in vitro* with allogeneic T cells. DCs from healthy donors could induce the production of IFN- $\gamma$  from T cells, indicating their capacity to orientate the response toward a type 1 response. In contrast, T cells stimulated with CLL-DCs increased their production of IL-10 without modifying IFN- $\gamma$  production, suggesting a predominant type 2 response (or even type 3, regulatory T cells, although this possibility was not investigated in our work).

Besides cytokine release, DCs provide T-cell stimulation through costimulatory molecules that amplify the signaling process. Engagement of CD28 by B7 molecules expressed on APCs allows T-cell activation, whereas T-cell receptor triggering in the absence of co-stimulation leads to T-cell anergy (43). DCs isolated from the PB of CLL patients expressed B7-2 (CD86) on their surface, but lacked almost completely the other costimulatory molecule B7-1 (CD80). Similarly, we could demonstrate that the presence of CLL cells during the maturation process of normal monocyte-derived DCs induces a marked decrease in the expression of CD80, without affecting, or even increasing, CD86 expression. CD80 and CD86 are members of the growing B7:CD28 family and, besides displaying different kinetics of expression, they might also differ in their costimulatory properties.

Although controversial, it has been suggested that the interaction between CD28 and CD80 stimulates Th1-responses, whereas CD86 would stimulate Th2-responses (44–49). Thus, the imbalance in the expression of CD80 and CD86 costimulatory molecules, with the predominance of the latter, is likely to play a role in the ability of DCs isolated from CLL patients to preferentially induce a type 2 response from allogeneic T cells.

The inhibitory effect of CLL cells on the generation of normal allogeneic DCs in Transwell cultures, although observed in a completely different experimental setting, suggests that the defects observed in the *in vivo* DC compartment are, at least in part, because of factors derived from the overwhelming leukemic clone. The impact of the neoplastic CLL cells on the *in vivo* DC compartment defects is also suggested by the possibility of generating apparently normal monocyte-derived DCs *in vitro*, even in patients with florid disease (34, 35).<sup>4</sup> However, if purified CD19+ autologous CLL cells are added to DC cultures, a marked inhibition of DC differentiation is observed (35).<sup>4</sup> The efforts to identify the soluble factors that could be responsible for the inhibitory action exerted by CLL cells on the growth and differentiation of DCs were partially successful. Neoplastic CLL cells are known to produce and release many cytokines, including IL-1, IL-6, IL-8, IL-10, tumor necrosis factor  $\alpha$ , tumor growth factor  $\beta$ , and VEGF among others (50–57). However, CLL patients are not homogeneous in their cytokine production profile. Analysis of IL-10 and IL-6 serum levels in CLL patients has shown a large array of heterogeneous values, from no production to significantly increased levels (33, 58, 59). It is possible that various factors may mediate an inhibitory effect on DCs, with a differentially relevant role in different patients. In our experiments, blocking of IL-6 activity was capable of reverting the inhibitory effect of CLL tumor cells in some patients, but not all, and IL-10 and VEGF also appeared to play a role in individual patients. It is likely that other factors need to be investigated and identified. The evidence, within the cases hereby studied, of a patient whose inhibitory activity could not be ascribed to any of the cytokines investigated additionally points to the above conclusion. For instance, macrophage-colony stimulating factor has been described as one of the factors capable of inhibiting DC maturation and function in solid tumors (13), and its production in CLL has been suggested (60). The possibility that this cytokine may play a role on CLL-DC activity may be worthy of investigation.

Taken together, our data point to the existence of peculiar defects within the circulating DC compartment of CLL patients, with a predominance of immature, type 2-driving DCs. These anomalies may have a relevant role in the genesis of the multiple T-cell abnormalities and in the severe immune impairment reported in these patients. We also demonstrated the ability of the neoplastic clone to generate *in vitro* in normal allogeneic DCs the same alterations observed *in vivo*. The mechanisms underlying this effect are not completely clarified, and more experiments will be necessary. A better definition of the factors mediating the inhibitory effect of neoplastic cells on DC development in cancer patients could be exploited in the near future to design strategies capable of reverting the DC impairment in these patients, with possible beneficial effects on their ability to mount efficient immune responses. This is particularly the case for conditions such as CLL, in which the abnormalities of the host immune compartment play a primary role in the clinical course of the disease and in the overall prognosis.

<sup>4</sup> E. Orsini, A. Guarini, S. Chiaretti, F. R. Mauro, and R. Foa, unpublished observations.



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