

# Dendritic Cells from Chronic Lymphocytic Leukemia Patients Are Normal Regardless of Ig V Gene Mutation Status

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Patients with B-type chronic lymphocytic leukemia (B-CLL) segregate into 2 subgroups based on the mutational status of the immunoglobulin (Ig) V genes and the patients in these subgroups follow very different clinical courses. To examine whether dendritic cells (DCs) generated from CLL patients can be candidates for immune therapy, we compared the phenotypic and functional capacities of DCs generated from patients of the 2 CLL subgroups (normal age-matched subjects [normal-DCs]). Our data show that immature DCs from B-CLL patients (B-CLL-DCs) have the same capacity to take up antigen as those from normal controls. Furthermore, B-CLL-DCs generated from the 2 CLL subgroups up-regulated MHC-II, CD80, CD86, CD83, CD40, and CD54 and down-regulated CD206 in response to stimulation with a cocktail of cytokines (CyC) and secreted increased levels of tumor necrosis factor  $\alpha$ , interleukin (IL)-8, IL-6, IL-12 (p70), and RANTES in a manner typical of mature normal-DCs. Interestingly, CD54 was significantly more up-regulated by CyC in B-CLL-DCs compared with normal-DCs. Except for CD54, no significant differences in surface molecule expression were observed between normal-DCs and B-CLL-DCs. B-CLL-DCs from both subgroups, including 6 patients with VH1-69, that usually fare poorly, presented tetanus toxoid to autologous T cells in vitro similar to normal-DCs. Our data show that DCs generated from the B-CLL subgroup with unmutated Ig V genes are functionally normal. These results are very promising for the use of DCs from patients with poor prognosis for immunotherapy.

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## INTRODUCTION

B cell chronic lymphocytic leukemia (B-CLL) is the most common leukemia in the Western world. Approximately 7500 people develop the disease annually and about 5000 die of the disease each year (1). Despite progress in therapy, to date there is no curative therapy for B-CLL (2). Therefore, the development of new strategies to attack re-emerging clones after classic therapy is necessary.

B-CLL patients can be divided into 2 subgroups based on immunoglobulin variable gene (Ig V) mutational status. Furthermore, others and we have shown that Ig mutation status (3,4) and CD38 expression (5) segregate patients into 2 subgroups that follow very different clinical courses (5,6).

Dendritic cells (DCs) have been used with some success in therapeutic cancer vaccines (7-9). For a DC-based tumor therapy to be successful, DCs from cancer patients must be able to take up tumor-specific antigens and present these in an immunostimulatory fashion to immunologically competent T cells. These cells, in turn, need to recognize and react to these antigens and mature to cytotoxic cells that destroy the tumor. Studies on the function of DC and T cells in B-CLL have provided some information about the capacity of these cells to carry out the functions necessary for a DC-based therapeutic approach to this disease.

Even though circulating blood DCs in B-CLL patients are defective (10), monocyte-derived DCs that can be generated from

B-CLL patients are functionally normal, aside from a reported abnormal cytokine pattern (11,12) and a reduction in some costimulatory molecules in patients with active disease (13). Furthermore, idiotype-pulsed DCs from B-CLL patients can induce leukemia-specific cytolytic T-cell responses in individuals with indolent but not aggressive disease (14), and T cells isolated from B-CLL patients can mount cytotoxic immune responses against allogeneic (15) and autologous (16) B-CLL cells.

Two key questions, however, have yet to be answered. First, what is the level of immune competence of the DCs in patients with unmutated V genes who fare poorly and are the patients most likely in need of therapy? Second, are the T cells from the 2 Ig V gene-defined B-CLL subgroups equally responsive to stimulation by autologous DC?

DCs that circulate in the blood or reside in solid tissues are immature (17,18). Such immature DC effectively endocytose antigens, although they express the accessory molecules needed for T cell activation at low levels. However, after antigen uptake and exposure to stimulatory signals, DCs mature. As a consequence of maturation, DCs express molecules that enable them to effectively bind and activate T cells in a manner that promotes immune responses (19), including those that are necessary for effective immunotherapy. In this study, we analyzed the phenotypic characteristics and cytokine profiles of immature and mature monocyte-derived DCs generated from B-CLL patients

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**Table 1. Clinical data of TT-vaccinated CLL patients<sup>a</sup>**

CLL Nr	Age/ sex	Rai stage	LDT	WBC/ Ald	Change of stage	Time to 1st treatment	Survival time after diagnosis
171	66/F	I	<12 mo	25.4/NA	To III at 7 y	Within 2 y	8+ y
334	54/M	II	~12 mo	38.4/30.91	To IV	At 2 y	4+ y
394	53/M	I	>12 mo	15.9/10.0	None	None	3+ y
521	65/M	II	NA	NA	NA	At 5 y	5+ y
559	48/M	0	<12 mo	14/6.5	Stage I in 3.5 y	At 3 y	6+ y
561	57/M	IV	NA	13.7/9.09	NA	From diagnosis	5+ y
569	51/M	II	NA	77.9/60.0	NA	NA	Alive <sup>b</sup>
349	59/F	0	<12 mo	10.0/NA	NA	NA	Alive <sup>b</sup>
124	68/M	0	>12 mo	12.6/59	To IV at 5 y	Within 3 y	~10 y
189	51/M	I	<12 mo	16.3/NA	To IV at 3 y	At 4 y	7+ y
567	55/F	0	>12 mo	11.4/6.4	None	None	4+ y

<sup>a</sup>LDT, lymphocyte doubling time; WBC, white blood cell count; Ald, absolute lymphocyte count at time of diagnosis; NA, not analyzed.

<sup>b</sup>One visit only.

(B-CLL-DC) with mutated or unmutated Ig V genes and compared these features with those from normal age-matched donors (normal-DC). Furthermore, we investigated the capacity of DCs from B-CLL patients to present the recall antigen tetanus toxoid (TT) to autologous T cells in vitro. We show that DCs from the 2 B-CLL subgroups are phenotypically and functionally similar to those generated from normal donors. In fact, mature B-CLL-DCs express higher levels of CD54, a molecule that is important in the generation of immunologically effective CD8<sup>+</sup> T cells (20), than normal DCs. These results are very promising for DC-based tumor therapy in B-CLL, because DCs generated from patients with poor prognostic markers are functional, and at least some fraction of the T cell population is not anergic.

## MATERIALS AND METHODS

### Patients and Healthy Donors

This study was approved by the Institutional Review Boards of North Shore University Hospital, Manhasset, NY, and Long Island Jewish Medical Center, New Hyde Park, NY. After obtaining informed consent, blood samples were collected from a large cohort of patients with clinical and laboratory features of B-CLL. For patients that received the TT vaccine, samples were obtained before and 2 to 3 wk after vaccination with TT. The mutational status of the Ig V genes was determined as previously described (20). Sequences with  $\geq 2\%$  difference in IgV<sub>H</sub> from the most similar germline gene were considered mutated ("mutated B-CLL"). Sequences with  $< 2\%$  difference were considered unmutated ("unmutated B-CLL"). Patients that lacked allelic exclusion at the H chain locus were considered mutated if either of the V<sub>H</sub> genes exhibited  $\geq 2\%$  difference from the most similar germline gene. Patients' clinical data are described in Table 1 and laboratory data in Table 2. Blood from normal volunteers was purchased from the Long Island Blood Services, Melville, NY, USA. These samples were tested and found negative for HIV-1, HTLV-1, HBV, and HBC antibodies and syphilis.

### Analysis of Peripheral Blood Mononuclear Cells (PBMC) from B-CLL Patients

PBMCs from both B-CLL patients and normal age-matched donors were separated by Ficoll-Hypaque density gradient centrifugation (Pharmacia LKB Biotechnology, New Brunswick, NJ, USA) and either used fresh or cryopreserved with a programmable cell-freezing machine (CryoMed, Mt. Clemens, MI, USA). PBMCs ( $1 \times 10^5$ ) were reacted for at least 20 min at 4°C in 100 mL phosphate-buffered saline (PBS)/5% fetal calf serum/0.1% sodium azide (staining buffer) with phycoerythrin (PE)-conjugated mAb specific for CD5 or CD38 (all from Beckton Dickinson Immunostaining Systems [BD], San Jose, CA, USA), in some cases fluorescein isothiocyanate (FITC)-conjugated IgG mAb specific for CD5 were used, APC-conjugated IgG specific for CD19, and PercP-conjugated IgG for CD3. Cells were then washed 4 times with staining buffer, fixed in 10% formaldehyde in PBS (pH 7.2-7.4), and examined by flow cytometry using a

**Table 2. Patient laboratory data**

CLL Nr	CD3 <sup>+</sup>	CD5 <sup>+</sup> / CD19 <sup>+</sup>	CD5 <sup>+</sup> / CD38 <sup>+</sup>	% VH mutation	VH gene usage
CLL 171	14	89	72	0	1-69
CLL 334	19	90	46	0	1-69
CLL 394	11	79	45	0	1-69
CLL 521	4	64	4	0	1-69
CLL 559	7	73	4	0	1-69
CLL 561	9	64	66	0	1-69
CLL 569	7	78	2	0.6	3-21
CLL 349	10	6	1	6.8	5-51
CLL 124	8	89	5	6, 5.7	1-03, 3-07
CLL 189	6	94	13	6.8, 0.3	4-34, 3-30
CLL 567	34	24	15	ND	ND

PBMCs from the group of CLL patients that received TT vaccination were analyzed for percentage of indicated cell populations before receiving the TT vaccine. The CD5<sup>+</sup>/CD38<sup>+</sup> cells are back gated on CD19<sup>+</sup> cells. Two cases (CLL 189 and CLL 124) lack allelic exclusion and the IgV gene sequence of CLL 567 could not be determined.

FACScan (BD). In all experiments, isotype controls were included using an appropriate PE- or FITC-conjugated irrelevant mAb of the same Ig class.

### Generation of Monocyte-Derived DCs

DCs generated from B-CLL patients are called B-CLL-DCs, and DCs generated from normal age-matched donors are called normal-DCs. PBMCs were incubated with anti-CD14 MACS beads (Miltenyi Biotech, Auburn, CA, USA), and CD14<sup>+</sup> cells were positively selected according to the manufacturer's instruction. CD14<sup>+</sup> cells were cultured in RPMI 1640 medium supplemented with 2 mM L-glutamine (GIBCO-BRL Life Technologies; Grand Island, NY, USA), 50  $\mu$ M 2-mercaptoethanol (Sigma, St. Louis, MO, USA), 10 mM HEPES (GIBCO-BRL), penicillin (100 U/mL)-streptomycin (100  $\mu$ g/mL) (GIBCO-BRL), and 5% human AB serum (Gemini Bio-Products, Woodland, CA, USA). Cultures were maintained for 7 d in 6-well trays ( $3 \times 10^6$  cells/well) and supplemented with 1000 U granulocyte-macrophage colony-stimulating factor per mL (Immunex, Seattle, WA, USA) and 200 U interleukin (IL)-4 per mL (R&D Systems; Minneapolis, MN, USA) at days 0, 2, 4, and 6.

At day 7 of culture, immature DCs were either left untreated ("immature," IM) or were stimulated with 100 ng/mL of LPS (*E. coli* serotype 026:B6, Sigma) or a cocktail of cytokines (CyC) (21) consisting of IL-6 (1000 U/mL), tumor necrosis factor  $\alpha$  (TNF- $\alpha$ ) (10 ng/mL), IL-1 $\beta$  (10 ng/mL) (all from R&D Systems), and prostaglandin E2 (PGE-2) (1  $\mu$ g/mL) (Sigma). In all experiments, DCs were analyzed 48 h after stimulation.

### Endocytosis

Immature DCs (day 7) were incubated on ice for at least 30 min and then incubated with Dextran-FITC (1 mg/mL) for 30 min at 4°C or 37°C. Cells were washed twice with cold medium, fixed, and analyzed by FACS for uptake of Dextran-FITC.

### Analysis of DC phenotype

DCs ( $1 \times 10^4$ ) were reacted for at least 20 min at 4°C in 100 mL staining buffer (PBS/5% fetal calf serum/0.1% sodium azide) with PE-conjugated mAb specific for CD206, CD54, HLA-DR (all from Beckton Dickinson Immunostaining Systems; San Jose, CA, USA), and CD83 (Immunotech-Beckman-Coulter; Marseille, France) or FITC-conjugated IgG mAb specific for CD80, and CD40 (all from Beckton Dickinson Immunostaining Systems; San Jose, CA). Cells were then washed 4 times with staining buffer, fixed in 3.7% formaldehyde in PBS (pH 7.2-7.4), and examined by flow cytometry using a FACScan. In all experiments, isotype controls were included using an appropriate PE- or FITC-conjugated irrelevant mAb of the same Ig class or subclass.

### Measurement of Cytokines and Chemokines

Forty-eight hours after activation, the production of cytokines and chemokines in cell culture supernatants was measured by enzyme-linked immunosorbent assay (Pierce Boston Technology Center, SearchLight™ Proteome Arrays Multiplex Sample Testing Services, Woburn, MA, USA).

### Isolation of T cells

T cells were purified from normal donors or B-CLL patients by negative selection using the Pan T cell isolation kit (Miltenyi) according to the manufacturer's instructions.

### Measurement of DNA Synthesis after Stimulation with TT

Immature DCs (day 6-7 in culture) were pulsed for 1.5 h with 10 to 50  $\mu$ g/mL of tetanus toxoid or left untreated (Staten Serum Institut, Cph. S., Denmark). Subsequently T cells were added to the DCs at a DC:T cell ratio of 1:10 and the co-cultures were stimulated with the cytokine cocktail described above to induce DC maturation (21). The co-cultures were plated at  $10^5$  cells per 96 well round bottom plate, incubated for 5 d, and pulsed with (<sup>3</sup>H)-thymidine (1  $\mu$ Ci/well) for the final 8 h of culture. Cell cultures were harvested onto glass fiber filters with a multiple automated sample harvester and the amount of isotope incorporation was determined by liquid scintillation  $\beta$ -emission. Responses were reported as mean cpm of (<sup>3</sup>H-TdR) incorporated by triplicate cultures.

### Statistical Analysis

For statistical comparison between groups, the unpaired Student *t* test was used with Welch correction.

## RESULTS

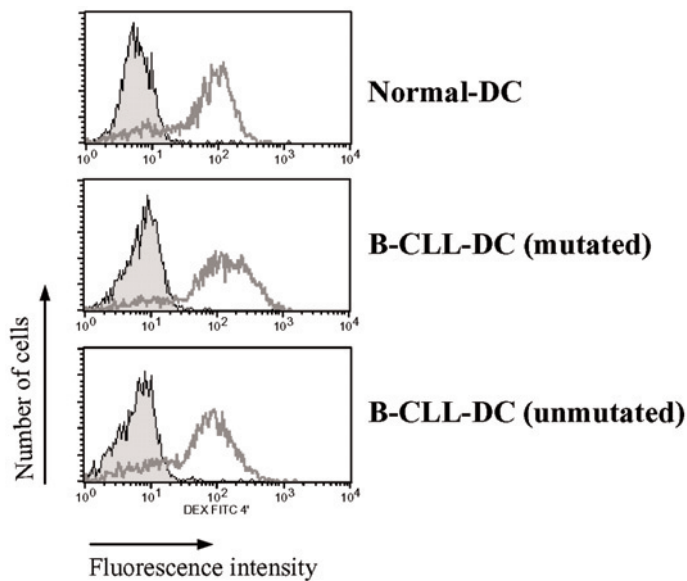
### Endocytosis of Soluble Antigen Is Similar between B-CLL-DCs and Normal Donors

To analyze endocytic capacity, dextran-FITC was used as a model soluble antigen. Endocytosis of dextran-FITC by immature B-CLL-DCs or normal-DCs was analyzed at 4°C (negative control) and 37°C (Figure 1). At 37°C, no difference was observed between the percentages of cells that had taken up dextran-FITC in B-CLL-DCs or normal-DCs, indicating that DCs generated from these patients have normal endocytic functions for soluble proteins.

### DCs Generated from B-CLL Patients Modulate Surface Molecules Similar to DCs from Normal Donors

To determine whether B-CLL-DCs can undergo phenotypic maturation typical of normal-DC, CyC (see Materials and Methods) or LPS was added to immature B-CLL-DCs and immature normal-DCs.

Increases in the cell surface density of MHCII, CD54, CD86, CD40, CD80, and CD83 expression and decrease in CD206 expression was observed (Figure 2A, B). For all molecules analyzed except CD54, the changes were not significantly different between B-CLL-DCs and normal-DCs as well as between the V gene mutation-defined B-CLL subgroups. Interestingly, CD54 was expressed at a significantly higher cell surface density ( $P = 0.001$ ) in mature B-CLL-DCs compared with mature normal-DCs (see Figure 2A, C). When the B-CLL patients were divided into the 2 subgroups, mature DCs from patients with mutated B-CLL showed significantly higher expression of CD54 compared with normal donors (see Figure 2C,  $P = 0.0034$ ). We did not have sufficient cases with unmutated B-CLL to make a



**Figure 1.** Endocytotic capacity of immature DCs generated from B-CLL patients and normal controls. Immature B-CLL-DCs or normal-DCs were incubated with 1 mg/mL of dextran-FITC for 30 min at 4°C (shaded histogram) or 37°C (nonshaded histogram). The cells were subsequently washed, fixed, and analyzed by FACS. One representative experiment is shown.

statistical analysis for CD54 and CD86, but the 2 cases that were analyzed showed similar expression levels to normal donors (data not shown).

### B-CLL-DCs Show a Typical Cytokine Profile

In addition to changes in surface molecule expression, secretion of inflammatory cytokines and chemokines characterizes mature DCs. B-CLL-DCs exposed to either CyC (see Figure 2A) or LPS (Figure 3B) showed increased production of TNF- $\alpha$ , IL-8, IL-6, and RANTES, which were not significantly different from normal-DCs. Very small increases, if any, were observed for IL-12 (p70) for both mature B-CLL-DCs and normal-DCs, probably due to the late time point (48 h) of measurement. Other groups have reported that immature B-CLL-DCs express higher levels of IL-10 than normal-DCs (11,12). Even though, in our studies, the spread of IL-12 and IL-10 secretion by immature DCs generated from different B-CLL cases was larger than in normal-DCs, IL-10 levels did not differ significantly between immature B-CLL-DCs and normal-DCs (see Figure 3C). IL-10 production increased in LPS-stimulated normal-DCs and less so in B-CLL-DCs, although the differences were not statistically significant. Furthermore, when the B-CLL patients were divided into 2 subgroups, no significant differences in production of IL-10, IL-8, IL-6, and IL-12 by immature and mature DCs were observed between either mutated B-CLL-DCs or unmutated B-CLL-DCs compared with normal-DCs. We could not study enough mutated cases to make a statistical analysis for TNF- $\alpha$  and RANTES production. However, no differences in the production of TNF- $\alpha$  and RANTES were observed between unmutated B-CLL-DCs and normal-DCs.

### DCs Generated from B-CLL Patients Induce a Proliferative Response to Tetanus Toxoid in Autologous T Cells

Because immature DCs from B-CLL patients show no defect in soluble antigen uptake, we analyzed whether they can present tetanus toxoid (TT) to autologous T cells that had been primed *in vivo*, thereby inducing a proliferative recall response.

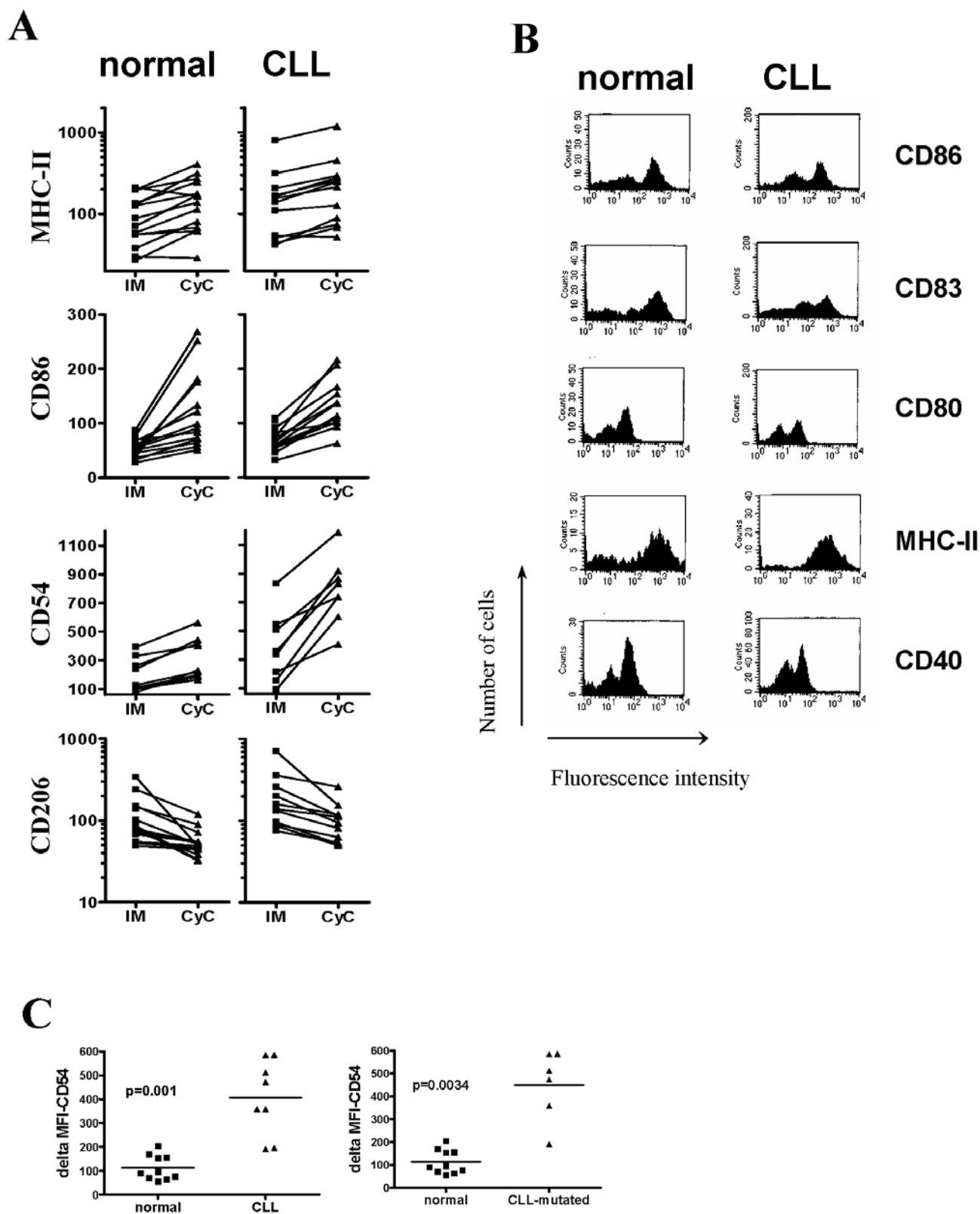
The B-CLL patients who received TT vaccination ( $n = 11$ ) consisted of 7 cases with unmutated IgV genes, 6 of which expressed  $V_H1-69$  with 0% mutation, and 1 that expressed  $V_H3-21$  with 0.6% mutations (Table 2). The usage of these  $V_H$  genes has been shown to be a marker for poor prognosis (5,6,22,23). Immature B-CLL-DCs ( $n = 11$ ) and normal-DCs ( $n = 6$ ) were pulsed with TT or media only and mixed with autologous T cells isolated 2 to 3 wk after the TT immunization. T cell proliferation was assessed after 5 d of culture (Table 3). Of the 7 unmutated-B-CLL cases, 5 showed a proliferation index between 1.5 and 6.6, whereas the other 2 did not show TT-specific T cell proliferation (CLL 394 and CLL 569). Of the 4 remaining cases, 3 were mutated B-CLL and 1 patient in whom IgV gene usage could not be determined (Table 2). All of those patients showed a TT response at the doses tested. Of the 6 normal donors, 5 showed a proliferation index between 1.2 and 3.2, and 1 (AG52) showed no TT-specific T cell proliferation (Table 3). However, no statistically significant differences were observed in the TT responses between all B-CLL cases as well as unmutated-B-CLL cases and normal donors. Not enough mutated B-CLL cases were available for statistical analysis.

### DISCUSSION

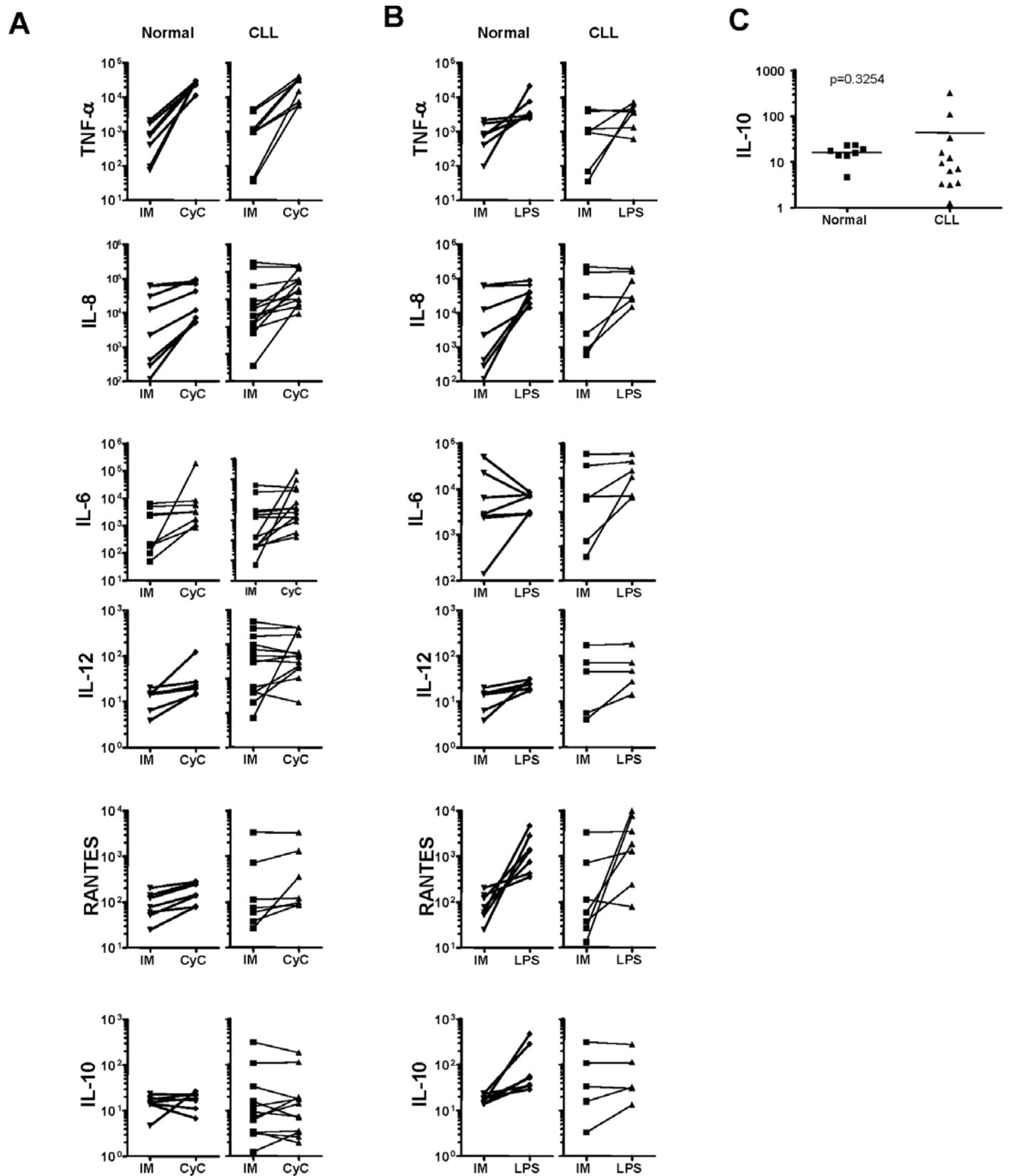
Our study shows that DCs can be generated from B-CLL patients and that they are phenotypically and functionally similar to those generated from normal age-matched donors. This is in accordance with recent reports (11,12,24). However the phenotypic and functional properties of DCs generated from CLL patients that express unmutated IgV genes, which usually have a more aggressive disease has not been investigated. We analyzed DCs generated from the 2 B-CLL subgroups based on IgV gene mutation status, which usually correlates with clinical outcome (5,6). Overall, DCs generated from B-CLL cases with mutated or unmutated IgV genes showed no functional or phenotypic differences.

Changes in surface expression of MHCII, CD86, CD83, CD40, and CD206 did not differ between B-CLL-DCs and normal DCs, independent of the IgV gene mutation status of the patients. In contrast to a recent report that showed reduced expression of CD80 and CD40 (13) in patients with active disease, we did not observe such differences in the B-CLL cases analyzed. This could reflect choice of patients and degrees of disease activity.

Interestingly, CD54 (intercellular adhesion molecule, ICAM1) was expressed at significantly higher levels in mature B-CLL-DCs compared with normal-DCs. This was also true when 6 mutated B-CLL-DCs were compared with normal-DCs; the number of unmutated cases was insufficient for statistical analysis, but the 2 cases that were analyzed were similar to normal DCs. When expressed by immature DCs, CD54 binds to integrins and stimulates CD4<sup>+</sup> T cells in the absence of antigen (20). In addition, block-



**Figure 2.** Phenotypic analysis of DCs generated from B-CLL patients and normal donors. A: Immature DCs from B-CLL patients and normal age-matched controls were stimulated with CyC (see Materials and Methods) for 48 h. Subsequently, cells were stained for the indicated surface antigens and analyzed by FACS. Mean fluorescence intensities (MFI) are depicted on the y-axis. B: Mature B-CLL-DCs and normal-DCs (48 h after stimulation with CyC) were analyzed for the expression levels of certain surface molecules. The result shown is representative of more than 4 independent experiments. C: Depicted are the delta MFI of CD54 in normal-DCs ( $n = 10$ ) and B-CLL-DCs, either all cases ( $n = 8$ ) on the left or cases with mutated IgV genes (CLL-mutated,  $n = 6$ ) on the right. The delta MFI represents the difference in mean fluorescence intensity between DCs matured in the presence of CyC and DCs cultured in medium only.



**Figure 3.** Cytokine profile of immature and mature DCs generated from B-CLL patients compared with normal donors. Cell culture supernatants from B-CLL-DCs and normal-DCs cultured in medium only (IM) and matured in the presence of CyC (A) or LPS (B) (100 ng/mL) were analyzed for the presence of the indicated cytokines and chemokines 48 h after stimulation. Levels shown are in pg/mL. Each line connecting 2 dots represents 1 donor. C: IL-10 levels (pg/mL) were measured in the cell culture supernatants of immature DCs generated from B-CLL patients ( $n = 12$ ) and normal donors ( $n = 8$ ). The horizontal bars indicate the mean levels of IL-10 protein.

**Table 3. Proliferative response of T cells to TT presented by autologous DCs**

	Proliferation index
Unmutated CLL	
CLL 171	1.9
CLL 334	6.6
CLL 394	1
CLL 521	1.5
CLL 559	2.8
CLL 561	1.6
CLL 569	1
Mutated CLL	
CLL 349	9.5
CLL 124	2.0
Lack of allelic exclusion	
CLL 189	1.8
Not determined	
CLL 567	2.5
Normal subjects	
AG 52	1
AG 81	2.2
AG 82	1.2
AG 83	2.7
AG 84	3.2
AG 85	3.2

The proliferation index represents a fold change increase in T-cell proliferation in TT pulsed DC-T cell cultures compared with nonpulsed cultures. 1 = no response. Eleven CLL patients and 6 normal subjects (AG) were analyzed.

ing CD54 and B7 on immature DCs can induce tolerogenic DCs (25). However in the mature state, CD54 provides co-stimulatory signals for CD8+ T cells (26) and contributes to the induction of high avidity CD8+ T cells (27). Therefore, the higher expression levels of CD54 in B-CLL-DCs might be beneficial for the induction of CTL responses.

Mature B-CLL-DCs secreted TNF- $\alpha$ , IL-6, IL-8, and RANTES at levels similar to normal-DCs. This was independent of the maturation stimulus used because both LPS and CyC showed comparable results. Increases in IL-6 and IL-8 production in mature DCs were not significantly different between the 2 B-CLL subgroups. The increases were similar to DCs generated from normal age-matched donors. Not enough mutated cases were available to compare TNF- $\alpha$  and RANTES production between mutated and unmutated cases. However, B-CLL-DCs from unmutated cases showed no difference in TNF- $\alpha$  and RANTES production compared with normal-DCs. The difference in IL-12 secretion between immature and mature DCs was small to negligible for both B-CLL-DCs and normal-DCs. This is most likely due to the timing of our analyses because cytokine secretion was measured 48 h after the induction of DC maturation and the peak of IL-12 secretion occurs at earlier timepoints (28). Also, 2 signals are required for high levels of IL-12 production (29).

IL-10 interferes with antitumor immune responses (30,31) and can also inhibit Th1 priming (32,33). In contrast to 2 other reports (11,12), we did not detect increased levels of spontaneous IL-10

secretion by immature B-CLL-DCs when compared with normal-DCs (see Figure 2C). Immature B-CLL-DCs showed a wider spread in IL-10 secretion than normal-DCs, but this was not statistically significant.

B-CLL patients with unmutated IgV genes, in particular  $V_H1-69$ , are likely to have an aggressive clinical course and require therapy (5,6). In addition, patients with leukemic cells that expressed  $V_H3-21$  also usually do poorly, regardless of mutation status (22,23). We analyzed 6  $V_H1-69^+$  patients and 1  $V_H3-21^+$  patient in our TT-study and found that DCs generated from these patients did not differ in their ability to induce an in vitro recall response to TT when compared with normal-DCs. This indicates that at least in vitro generated DCs and TT-specific T cells from this group of patients are functionally normal. When mature DCs instead of immature DCs were pulsed with TT, no TT-specific response was induced in the autologous T cells (data not shown). Also the absence of cocktail-induced maturation after the TT-pulse led to a lack in T cell stimulation (data not shown). This confirms that DCs must be immature at the time of the TT pulse to take up antigen and that subsequent maturation is required for T cell stimulation. These issues will have to be considered for in vivo immunization as well. Taken together, our results suggest that DCs generated from B-CLL patients with bad clinical prognosis as determined by the Ig V gene mutation status are feasible candidates for tumor immunotherapy.

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## REFERENCES

- Landis SH, Murray T, Bolden S, Wingo PA. (1999) Cancer statistics, 1999. *CA Cancer J. Clin.* 49:8-31.
- Dighiero G, Binet JL. (2000) When and how to treat chronic lymphocytic leukemia. *N. Engl J. Med.* 343:1799-801.
- Schroeder HWJ, Dighiero G. (1994) The pathogenesis of chronic lymphocytic leukemia: analysis of the antibody repertoire [see comments]. *Immunology Today* 15:288-94.
- Fais F et al. (1998) Chronic lymphocytic leukemia B cells express restricted sets of mutated and unmutated antigen receptors. *J. Clin. Invest.* 102:1515-25.
- Damle RN et al. (1999) Ig V gene mutation status and CD38 expression as novel prognostic indicators in chronic lymphocytic leukemia. *Blood* 94:1840-7.
- Hamblin TJ, Davis Z, Gardiner A, Oscier DG, Stevenson FK. (1999) Unmutated Ig V(H) genes are associated with a more aggressive form of chronic lymphocytic leukemia [see comments]. *Blood* 94:1848-54.

7. Gapin L et al. (1998) Antigen presentation by dendritic cells focuses T cell responses against immunodominant peptides: studies in the hen egg-white lysozyme (HEL) model. *J. Immunol.* 160:1555-64.
8. Raje N et al. (1999) Bone marrow and peripheral blood dendritic cells from patients with multiple myeloma are phenotypically and functionally normal despite the detection of Kaposi's sarcoma herpesvirus gene sequences. *Blood* 93:1487-95.
9. Timmerman JM, Levy R. (1999) Dendritic cell vaccines for cancer immunotherapy. *Annu. Rev. Med.* 50:507-29.
10. Orsini E, Guarini A, Chiaretti S, Mauro FR, Foa R. (2003) The circulating dendritic cell compartment in patients with chronic lymphocytic leukemia is severely defective and unable to stimulate an effective T-cell response. *Cancer Res.* 63:4497-506.
11. Vuillier F et al. (2001) Functional monocyte-derived dendritic cells can be generated in chronic lymphocytic leukemia. *Br. J. Haematol.* 115:831-44.
12. Rezvany MR et al. (2001) Dendritic cells in patients with non-progressive B-chronic lymphocytic leukemia have a normal functional capability but abnormal cytokine pattern. *Br. J. Haematol.* 115:263-71.
13. Orsini E et al. (2004) Phenotypic and functional characterization of monocyte-derived dendritic cells in chronic lymphocytic leukemia patients: influence of neoplastic CD19 cells in vivo and in vitro. *Br. J. Haematol.* 125:720-8.
14. Vuillier F et al. (2003) Idiotype-pulsed dendritic cells are able to induce antitumor cytotoxic CD8 cells in chronic lymphocytic leukemia. *Br. J. Haematol.* 120:243-50.
15. Krackhardt AM et al. (2002) T-cell responses against chronic lymphocytic leukemia cells: implications for immunotherapy. *Blood* 100:167-73.
16. Goddard RV, Prentice AG, Copplestone JA, Kaminski ER. (2001) Generation in vitro of B-cell chronic lymphocytic leukemia-proliferative and specific HLA class-II-restricted cytotoxic T-cell responses using autologous dendritic cells pulsed with tumor cell lysate. *Clin. Exp. Immunol.* 126:16-28.
17. O'Doherty U et al. (1994) Human blood contains two subsets of dendritic cells, one immunologically mature and the other immature. *Immunology* 82:487-93.
18. Weissman D et al. (1995) Three populations of cells with dendritic morphology exist in peripheral blood, only one of which is infectable with human immunodeficiency virus type 1. *Proc. Natl. Acad. Sci. U.S.A.* 92:826-30.
19. Banchereau J, Steinman RM. (1998) Dendritic cells and the control of immunity. *Nature* 392:245-52.
20. Real E et al. (2004) Immature dendritic cells (DCs) use chemokines and intercellular adhesion molecule (ICAM)-1, but not DC-specific ICAM-3-grabbing nonintegrin, to stimulate CD4+ T cells in the absence of exogenous antigen. *J. Immunol.* 173:50-60.
21. Jonuleit H et al. (1997) Pro-inflammatory cytokines and prostaglandins induce maturation of potent immunostimulatory dendritic cells under fetal calf serum-free conditions. *Eur. J. Immunol.* 27:3135-42.
22. Tobin G et al. (2002) Somatically mutated Ig V(H)3-21 genes characterize a new subset of chronic lymphocytic leukemia. *Blood* 99:2262-4.
23. Tobin G et al. (2003) Chronic lymphocytic leukemias utilizing the VH3-21 gene display highly restricted Vlambda2-14 gene use and homologous CDR3s: implicating recognition of a common antigen epitope. *Blood* 101:4952-7.
24. Vuillier F, Dighiero G. (2003) Monocyte-derived dendritic cells in chronic lymphocytic leukemia. *Leuk. Lymphoma* 44:1267-73.
25. Wang Q et al. (2003) Anti-ICAM-1 antibody and CTLA-4Ig synergistically enhance immature dendritic cells to induce donor-specific immune tolerance in vivo. *Immunol. Lett.* 90:33-42.
26. Deeths MJ, Mescher MF. (1999) ICAM-1 and B7-1 provide similar but distinct costimulation for CD8+ T cells, while CD4+ T cells are poorly costimulated by ICAM-1. *Eur. J. Immunol.* 29:45-53.
27. Oh S et al. (2003) Selective induction of high avidity CTL by altering the balance of signals from APC. *J. Immunol.* 170:2523-30.
28. Kalinski P, Schuitmaker JH, Hilkens CM, Wierenga EA, Kapsenberg ML. (1999) Final maturation of dendritic cells is associated with impaired responsiveness to IFN-gamma and to bacterial IL-12 inducers: decreased ability of mature dendritic cells to produce IL-12 during the interaction with Th cells. *J. Immunol.* 162:3231-6.
29. Snijders A, Kalinski P, Hilkens CM, Kapsenberg ML. (1998) High-level IL-12 production by human dendritic cells requires two signals. *Int. Immunol.* 10:1593-8.
30. Zhu LX et al. (2000) Delta-9-tetrahydrocannabinol inhibits antitumor immunity by a CB2 receptor-mediated, cytokine-dependent pathway. *J. Immunol.* 165:373-80.
31. Gray CP, Franco AV, Arosio P, Hersey P. (2001) Immunosuppressive effects of melanoma-derived heavy-chain ferritin are dependent on stimulation of IL-10 production. *Int. J. Cancer* 92:843-50.
32. Powrie F, Menon S, Coffman RL. (1993) Interleukin-4 and interleukin-10 synergize to inhibit cell-mediated immunity in vivo. *Eur. J. Immunol.* 23:2223-9.
33. Contti P et al. (2003) IL-10, an inflammatory/inhibitory cytokine, but not always. *Immunol. Lett.* 86:123-9.