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Vδ1 T Lymphocytes from B-CLL Patients Recognize ULBP3 Expressed on Leukemic B Cells and Up-Regulated by *Trans*-Retinoic Acid

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ABSTRACT

We analyzed 38 untreated patients with chronic lymphocytic leukemia of B-cell type (B-CLL): 24 low-, 8 intermediate-, and 6 high-risk stage. In 15 patients (13 low risk and 2 intermediate risk), circulating Vδ1 T lymphocytes were significantly increased (100 to 300 cells/μL) compared with most intermediate, all high-risk stage, and 15 healthy donors (50 to 100 cells/μL). We studied these Vδ1 T lymphocytes and observed that they proliferated *in vitro* and produced tumor necrosis factor α or IFN-γ in response to autologous leukemic B cells but not to normal lymphocytes. However, they were unable to kill resting autologous B cells, which lack the MHC-related MIC-A antigen and express low levels of the UL16-binding protein (ULBP) 3 and undetectable levels of ULBP1, ULBP2, and ULBP4. All these molecules are reported ligands for the NKG2D receptor, which is expressed by γδ T cells and activates their cytolytic function. The Vδ1 T lymphocytes studied were able to lyse the ULBP3$^+$ C1R B-cell line upon transfection with MIC-A. More importantly, they also lysed autologous B-CLL cells when transcription and expression of MIC-A or up-regulation of ULBP3 were achieved either by activation or by exposure to *trans*-retinoic acid. The NKG2D receptor expressed on Vδ1 T cells was involved in the recognition of B-CLL. Finally, in six patients with low numbers of circulating Vδ1 T cells and undetectable ULBP3, the disease progressed over 1 year, whereas no progression occurred in patients with high Vδ1 T lymphocytes and detectable/inducible ULBP3. These data suggest that Vδ1 T lymphocytes may play a role in limiting the progression of B-CLL.

INTRODUCTION

Chronic lymphocytic leukemia of B-cell type (B-CLL) is the most frequent adult leukemia in Western countries and many patients present an aggressive disease resistant to chemotherapy (1). This makes of interest the dissection of the mechanisms and the identification of selected populations that might be involved in host defense against this type of cancer. There is increasing evidence that αβ and γδ T lymphocytes make distinct contributions to anticancer surveillance (2–6). Indeed, among γδ T lymphocytes, the circulating Vδ2 subset is capable of killing myeloma and Burkitt lymphoma cells (7–10), whereas the Vδ1 subset, which is mainly located in the mucosal associated lymphoid tissue, has been implied in the defense against epithelial cancers (8, 11, 12). It has been reported that in some hematologic malignancies circulating γδ T lymphocytes are increased: Vδ1 T cells have been found with high frequency in disease-free survivors of acute leukemia after bone marrow transplantation, whereas an expansion of Vδ2 T lymphocytes has been described in multiple myeloma (10, 13). Little is known, thus far, on the possibility that Vδ2 and Vδ1 T cells play a role in limiting the onset and spreading of B-CLL.

The Vδ2 subset recognize low molecular weight, phosphate-containing, non peptide antigens, without the need of antigen processing and presentation by conventional MHC molecules (14–17). Moreover, evidence has been provided that Vδ1 T cells can recognize the human MHC class I-related molecules MIC-A and MIC-B expressed by intestinal epithelial cells and that several epithelial tumors are infiltrated by Vδ1 lymphocytes that kill MIC-A– or MIC-B–expressing tumor cells (18, 19). Notably, MIC-A expression has been described in some leukemic cell lines (20). MIC-A and MIC-B have been described as ligands for the natural killer cell receptor NKG2D, which is expressed by cytotoxic effector cells, including γδ T lymphocytes (21). More recently, additional counterreceptors for NKG2D have been found, that is the UL16-binding protein (ULBP) 1–4 molecules that bind to the cytomegalovirus glycoprotein UL16 (22). Engagement of NKG2D by these ligands leads to the activation of cytotoxic effector lymphocytes and to the enhancement of T-cell receptor-mediated effector functions in a subset of γδ T cells (21–23). In mice, γδ T lymphocytes were shown to kill tumor cells through the interaction between NKG2D and the murine retinoic acid early inducible (RAE-1) protein, a MHC-related molecule like the human MIC-A and MIC-B (4, 24).

In this article, we provide evidence that (*a*) circulating Vδ1 T lymphocytes are increased in a proportion of B-CLL patients and proliferate *in vitro* in response to autologous leukemic B cells; (*b*) Vδ1 T lymphocytes produce tumor necrosis factor α (TNF-α) and IFN-γ upon coculture with autologous B-CLL cells; and (*c*) antitumor cell killing can be obtained by inducing transcription and surface expression of MIC-A or up-regulation of ULBP3 upon exposure of B-CLL cells to *trans*-retinoic acid.

MATERIALS AND METHODS

Patients. Thirty-eight untreated patients with B-CLL were studied, provided informed consent, at the Clinical Hematology Division (Department of Hematology and Oncology, University of Genoa, Genoa, Italy). The patients met the diagnostic criteria of the National Cancer Institute Working Group (25) and were staged according to the Rai-modified criteria (26) as low risk, intermediate risk, and high risk. Their clinical and immunophenotypic features are summarized in Table 1. Fifteen healthy subjects, matched for sex and age, were also studied.

Monoclonal Antibodies and Reagents. The anti-Vδ1 monoclonal antibody (mAb) A13 and anti-Vδ2 mAb (BB3 or γδ123R3 clones, both IgG1) were prepared as described previously (27). The anti-αβ TCR BMA031 (IgG1) and anti-Vδ1 mAb MCA2080 (IgG1) were obtained from Serotec (Cergy Saint-Christophe, France). The anti-CD38 mAb (clone HT7, IgG1) was from BD PharMingen Europe (Milan, Italy) and the anti-NKG2D (IgG1) from R&D System Europe (Oxon, United Kingdom). The anti-HLA class-I W6/32 (IgG2a)-producing hybridoma was purchased from the American Type Culture Collection (Manassas, VA), and W6/32 mAb was purified by affinity chromatography. The anti-MIC-A mAbs AMO1 and BAMO3 were from Immatics Biotechnologies (Tubingen, Germany), and the anti-ULPB mAbs (anti-ULBP1 M291, anti-ULBP2 M311, anti-ULBP3 M551, anti-ULBP4 M478) were

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Table 1 *Characteristics of B-CLL patients*

Patients *	Sex	Age	Year †	Stage ‡	Lymphocytes (cells × $10^3/\mu L^{-1}$) §	Vδ1 (cells/μL) ¶	Immunophenotype (at diagnosis) ‖	IgV/H mutations **
01	M	62	2002	LR	19.3	248	$CD38^-$	Mutated
02	F	61	2000	LR	16.0	109	$CD38^-$	ND
03	F	58	2000	LR	10.9	336	$CD38^-$	Mutated
04	F	65	2000	LR	19.9	219	$CD38^-$	Mutated
05	M	62	2002	IR	15.3	168	$CD38^-$	Unmutated
06	F	55	2003	IR	8.8	231	$CD38^-$	Unmutated
07	F	63	1998	LR	10.6	150	$CD38^-$	ND
08	M	66	1999	LR	12.5	106	65% $CD38^+$	ND
09	F	69	2001	LR	10.8	58	42% $CD38^+$	ND
10	M	67	2000	LR	18.0	75	31% $CD38^+$	ND
11	M	68	2000	IR	10.9	53	51% $CD38^+$	Mutated
12	F	62	1999	LR	14.9	114	$CD38^-$	Mutated
13	M	57	2003	IR	12.7	86	39% $CD38^+$	Unmutated
14	F	63	1992	LR	8.5	19	$CD38^-$	ND
15	M	73	2001	LR	13.1	122	$CD38^-$	ND
16	F	55	2002	LR	12.7	1	52% $CD38^+$	Unmutated
17	M	66	2001	LR	9.8	20	40% $CD38^+$	Unmutated
18	M	59	1994	LR	9.4	7	58% $CD38^+$	ND
19	M	63	2000	IR	12.0	80	80% $CD38^+$	Unmutated
20	F	57	2001	LR	8.1	15	$CD38^-$	ND
21	F	63	1997	LR	11.5	138	$CD38^-$	Mutated
22	M	67	2001	LR	33.4	75	38% $CD38^+$	Unmutated
23	F	51	1994	LR	15.6	110	$CD38^-$	ND
24	M	60	1998	IR	19.0	10	$CD38^-$	Unmutated
25	F	64	1999	LR	11.5	207	$CD38^-$	Mutated
26	F	65	2002	LR	19.2	199	$CD38^-$	Mutated
27	M	61	2001	LR	21.1	10	30% $CD38^+$	ND
28	F	56	1999	LR	46.8	128	$CD38^-$	Mutated
29	M	60	2001	LR	3.0	2	$CD38^-$	ND
30	M	65	2000	LR	18.0	4	30% $CD38^+$	Unmutated
31	M	50	1999	LR	7.0	3	60% $CD38^+$	Unmutated
32	F	61	1994	IR	5.2	4	36% $CD38^+$	Unmutated
33	F	83	1998	HR	10.9	21	$CD38^-$	Unmutated
34	M	75	1994	HR	9.8	11	40% $CD38^+$	ND
35	M	67	2001	HR	33.4	66	60% $CD38^+$	ND
36	M	62	2000	HR	18.9	6	56% $CD38^+$	Unmutated
37	M	48	2001	HR	41.5	10	$CD38^-$	Unmutated
38	M	65	2002	HR	46.8	4	80% $CD38^+$	Unmutated

* Chemonaive patients who never had received chemotherapy.
† Year of diagnosis and staging.
‡ Stage defined according to the Rai modified classification (26): LR, low risk; IR, intermediate risk; HR, high risk.
§ Lymphocyte count at the time of the study.
¶ Absolute number of peripheral blood Vδ1 T lymphocytes/μl.
‖ Percentage of CD38+ leukemic B cells is indicated.
** Immunoglobulin mutation was evaluated as described previously (29, 31). The sequences with a germ line homology 98% or higher were considered unmutated and those with a homology < 98% mutated (31, 35). ND, not done.

kindly provided by Amgen (Seattle, WA). Phytohemagglutinin (PHA), pokeweed mitogen (PWM), all-*trans*-retinoic acid (ATRA) and murine immunoglobulin were from Sigma Chemicals Co. (St. Louis, MO) and recombinant interleukin 2 was from Pepro Tec EC (London, United Kingdom).

Isolation and Culture of γδ T-Cell Populations. Peripheral blood mononuclear cells (PBMCs) from B-CLL patients and healthy donors were isolated by Ficoll-Hypaque gradient. Highly purified $CD3^+$ $\gamma\delta^+$ T cells were obtained by negative immunodepletion with anti-CD14, anti-CD4, and anti-CD8–coated magnetic microbeads (StemCell Technologies, Vancouver, British Columbia, Canada). To obtain Vδ1 or Vδ2 T-cell lines, $CD4^-CD8^-$ cells were seeded under limiting dilution conditions in 96-well U-bottomed microplates (Greiner Labortecknic, Nurtingen, Germany) and cultured in RPMI 1640 supplemented with 200 mmol/L L-glutamine 10% of FCS, 1 μg/mL PHA, 25 units/mL recombinant interleukin 2, and 10^5 irradiated (3000 rad) autologous PBMCs as feeder cells as described previously (27). Vδ1 cell lines were $A13^+MCA2080^+$, Vδ2 cell lines were $BB3^+$ and all were BMA01 negative (data not shown). Autologous B cells were obtained by negative depletion with anti-CD14, anti-CD4, and anti-CD8–coated magnetic microbeads (StemCell Technologies) and were >98% pure as assessed by CD20 staining. Proliferation of Vδ1 or Vδ2 T cells to autologous B-CLL PBMCs *versus* normal irradiated resting or PWM-activated PBMCs was evaluated by counting the number of $A13^+MCA2080^+$ (Vδ1) or $BB3^+$ (Vδ1) cells at different times (1, 2, 3, 5, 7, and 10 days) of culture. After 48 hours (day 2), supernatants were recovered from the cocultures for the measurement of TNF-α and IFN-γ. At day 5, 25 units/mL recombinant interleukin 2 were added to the cultures.

Immunofluorescence and Cytofluorometric Analysis. Single or double immunofluorescence on peripheral whole blood of healthy donors and B-CLL patients was performed with the various mAbs labeled with the fluorochromes Alexafluor 488 or 594 contained in the Zenon Tricolor Labeling kit for mouse IgG1, IgG2a, or IgG2b (Molecular Probes Europe BV, Leiden, the Netherlands). Immunofluorescence staining of cultured cells was performed as described elsewhere (27). Control aliquots were stained with Alexafluor-labeled isotype-matched irrelevant mAbs. Samples were run on a flow cytometer (FACScan, Becton Dickinson, San Jose, CA) equipped with an argon ion laser exciting phycoerythrin at 488 nm. Data were analyzed with CellQuest computer program and are expressed as log red fluorescence intensity (arbitrary units, a.u.) *versus* log green fluorescence intensity (a.u.) or *versus* number of cells or as mean fluorescence intensity (a.u.). Calibration was assessed with CALIBRITE particles (Becton Dickinson) with the AutoCOMP computer program.

Cytotoxicity Assay. Cytolytic activity of Vδ1 or Vδ2 T cell lines from B-CLL patients was analyzed in a 4-hour ^{51}Cr-release assay against the B lymphoma cell lines Daudi and Raji or against the autologous or allogenic tumor B cells, labeled with ^{51}Cr, at an E:T ratio of 40:1 to 10:1, in a final volume of 200 μL of culture medium in V-bottomed microwells (27). The C1R B lymphoma cell line, wild type or transfected with the cDNA coding for MIC-A and expressing the ULBP3 molecule (28), was also used as target. One hundred microliters of supernatant were counted in a gamma counter, and percentage of ^{51}Cr-specific release was calculated as described previously (27). Some experiments were done with, as targets, the autologous leukemic B cells pretreated for 3 days with 1 μg/mL PWM or with 10 μg/mL ATRA for 24 hours; in some samples, the effector cells were exposed to saturating amounts (3 μg/mL) of the anti-NKG2D, the anti-MIC-A, the anti-ULBP1 or 3

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or the anti-HLA class-I mAbs before start the cytotoxicity assay. Statistical analysis was performed with the Student *t* test.

Evaluation of mRNA for MIC-A, MIC-B, and ULBPs. Total RNA was prepared from PBMCs with TRIpure (Sigma Chemicals Co.) and reverse transcribed with the RT kit from Amplimedical S.p.A. (Bioline Division, Milan, Italy). The resulting cDNA was amplified by PCR with specific primer pairs in 32 cycles at 95°C for 1 minute and 60°C and 72°C for 1 minute. Oligonucleotide sequences (forward and reverse) were as follows: β-actin, 5′-CATACTCCTGCTTGCTGATCC-3′ and 5′-ACTCCATCATGAAGTGTGACG-3′; MIC-A, 5′-CCTTGGCCATCAACGTCAGG-3′ and 5′-CCTCTGAGGCCTCRCTGCG-3′; MIC-B, 5′-ACCTTGGCTATGAACGTCACA-3′ and 5′-CCCTCTGAGACCTCGCTGCA-3′; ULBP1, 5′-GTACTGGGAACAAATGCTGGAT-3′ and 5′-AACTCTCCTCATCTGCCAGCT-3′; ULBP2 5′-TTACTTCTCAATGGGAGACTGT-3′ and 5′-TGTGCCTGAGGACATGGCGA-3′; and ULBP3, 5′-CCTGATGCACAGGAAGAAGAG-3′ and 5′-TATGGCTTTGGGTTGAGCTAAG-3′; amplicons were examined on 2% agarose gel for correct size (28). Images were acquired by Chemi 550 (AlphaInnotech, San Leonardo, CA) and analyzed by Gel Pro Analyzer 3.1 (Media Cybernetics, Silver Spring, MD). Results are expressed as fold increase vs. basal level.

Determination of Mutational Status of IgV_H Genes. In 24 of 38 patients the analysis of IgV_H genes mutational status was performed on cDNA as described previously (29). After determining the IgV_H gene family used by the leukemic cells, the V_H gene sequences were determined by amplifying 2.5 μL of cDNA by PCR with the appropriate sense V_H leader primer in combination with the appropriate antisense C_H primer. The PCR products were sequenced directly after purification with PRC Preps (Promega, Madison, WI) with an automated DNA sequencer (Applied Biosystems, Foster City, CA) Sequences were compared with those present both in the V BASE sequence directory and in the IMGT/V-QUEST database (30).[7,8] The sequences with a germ line homology 98% or higher were considered unmutated and those with a homology < 98% mutated (30, 31).

ELISA for the Measurement of Soluble MIC-A, TNF-α, and IFN-γ. Soluble MIC-A was measured in the serum of B-CLL patients or in the supernatant of PWM- or ATRA-stimulated B-CLL cells or PHA-activated T cells after 48 hours of culture by ELISA with the commercial kit developed by Immatics Biotechnologies. Plates were coated with the capture anti-MIC-A mAb AMO-1 at 5 μg/mL in PBS overnight at 4°C and blocked with 7% PBS-BSA for 2 hours at 37°C. Standard (recombinant MIC-A*04) and samples (supernatant or patients' sera, diluted 1:3 in 7% BSA-PBS) were added and incubated 2 hours at 37°C. After washing, the detection anti-MIC-A mAb BAMO3 was added at 2 μg/mL for 2 hours at 37°C, followed by the horseradish peroxidase-conjugated antimouse IgG2a antiserum (Sigma Chemicals Co.) at 1:10,000 dilution for 1 hour at 37°C. Plated were then developed with the specific substrate (Sigma Chemicals Co.) and read at 450 nm wavelength (28). TNF-α or IFN-γ was measured by ELISA in the supernatant of Vδ1 or Vδ2 T cells (10^6 cells) from 10 patients, after 48 hours of culture with B-CLL or normal B cells (10^6 cells), with the commercial kits purchased from PeproTec.

RESULTS

Vδ1 T Lymphocytes from B-CLL Patients with Stable Disease Are Increased in Peripheral Blood, Proliferate, and Produce TNF-α and IFN-γ in Response to Autologous Leukemic B Cells. We found that in 15 of 38 B-CLL patients (13 low risk and 2 intermediate risk), circulating Vδ1 T lymphocytes, which usually belong to the resident population of γδ T cells and are barely detectable in peripheral blood, were significantly increased (mean value, 150; range, 100 to 300 cells/μL; Table 1) compared with 15 healthy donors matched for sex and age (mean value, 54; range, 50 to 100 cells/μL; $P < 0.05$; data not shown in tables or figures). In 13 of these 15 patients, B cells were $CD38^-$, and in seven of the nine analyzed, the IgV_H genes were mutated (Table 1). In the same patients, Vδ2 T cells were only slightly increased (120 to 200 *versus* 100 to 150 cells per μL of healthy donors), not reaching statistical significance (data not shown). The remaining 23 patients had a normal or low Vδ1 count (12 low risk, 5 intermediate risk, and 6 high risk), their B cells were mostly $CD38^+$ (17 of 23), and IgV_H genes were unmutated in more than half (13 of 23) of the patients (Table 1). More specifically, five of six of the high-risk patients were $CD38^+$, four were unmutated, and none had increased Vδ1 T cells (Table 1).

When purified, Vδ1 T lymphocytes were cultured in the presence of irradiated autologous B-CLL PBMCs as feeder cells, a strong proliferation of the Vδ1 T cell subset was observed in all of the patients, reaching a 10-fold increase in cell number at day 5 of culture and a 20-fold increase after 5 additional days of culture in recombinant interleukin 2 (Fig. 1*A*). This effect was more evident (100-fold increase) when PWM-activated autologous PBMCs were used as stim-

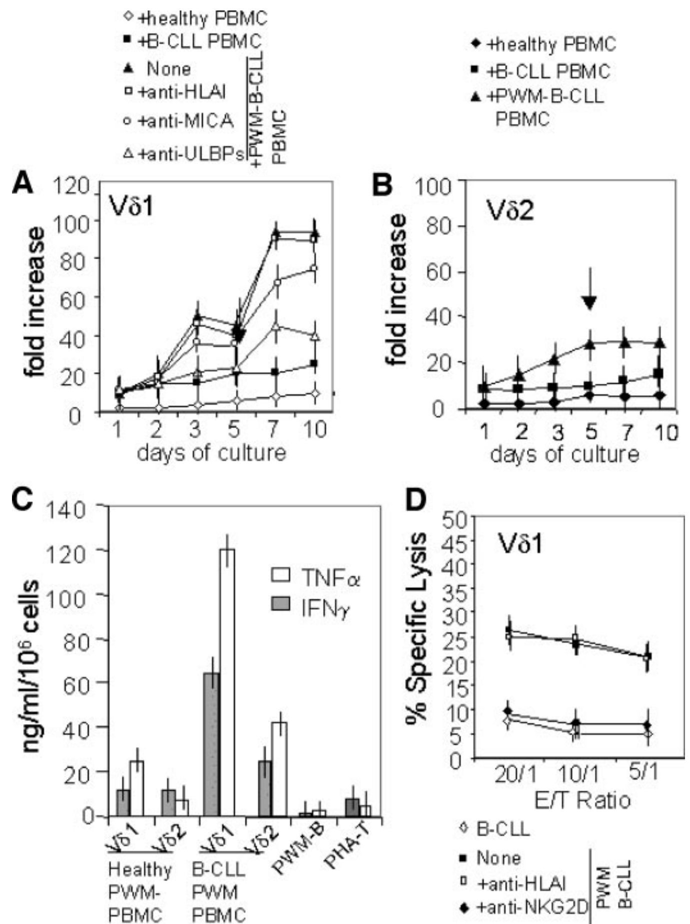

Fig. 1. Vδ1 T lymphocytes from B-CLL patients proliferate, produce TNF-α and IFN-γ and exert cytolytic activity in response to autologous activated PBMCs. Vδ1 (*A*) or Vδ2 (*B*) T lymphocytes were separated from all of the 15 patients with increased circulating γδ T cells and cocultured with irradiated autologous PBMCs (>80% represented by leukemic B cells), untreated or treated for 48 hours with PWM, or with PBMCs from healthy donors, as feeder cells, in the absence (none) or presence of the indicated mAbs (5 μg/mL). At day 5, 25 units/mL recombinant interleukin 2 were added (*arrows*) for the following 5 days. At the indicated time points, cells were recovered, counted, and checked for Vδ1 or Vδ2 expression, and results are expressed as mean fold-increase in cell number. *C*. TNF-α or IFN-γ were measured by ELISA in the supernatant of Vδ1 or Vδ2 T cells from 15 B-CLL patients after 48 hours of culture with B-CLL or healthy PBMCs; results are expressed as ng/mL/10^6cells (mean ± SD from experiments performed with cells obtained from 15 different patients). *D*. Vδ1 T-cell lines obtained from ten B-CLL patients were tested in 4-hour ^{51}Cr release assay against resting or PWM-activated autologous neoplastic B cells (B-CLL) at the indicated E:T ratios. The specific anti-NKG2D or the anti-HLA-I mAb were used at the concentration of 5 μg/mL. None: no mAb added. Results are expressed as percent specific lysis calculated as described in Materials and Methods and are the mean ± SD from experiments performed with cell lines obtained from 10 patients.

[7] Internet address: http://www.mrc-cpe.cam.ac.uk/imt-doc/vbase-home-page.htlm.
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ulators; on the contrary, no evident proliferation occurred when Vδ1 T cells were cultured in the presence of PBMCs of healthy donors, irrespective of their activation (Fig. 1*A*). As for Vδ2 T lymphocytes, the number of cells recovered at different time points increased slowly and independently of the stimulator, *i.e.,* in coculture with either B-CLL or healthy PBMCs. Also, their proliferative response to PWM-activated B-CLL PBMCs was lower than that observed for Vδ1 T lymphocytes (Fig. 1*B versus* Fig. 1*A*).

These data suggest that in B-CLL patients the expansion of Vδ1 T cells might be attributable to signals delivered by autologous leukemic B cells, possibly through molecules up-regulated at B-cell surface by activation. The *in vitro* proliferation of Vδ1 T lymphocytes was inhibited by a mixture of anti-ULBP mAbs but not by anti-HLA-I mAbs and only slightly by anti-MIC-A mAbs (Fig. 1*A*). Importantly, we found that γδ T cells from B-CLL patients can produce cytokines with potential antitumor activity: in particular, Vδ1 T lymphocytes produced considerable amounts of both TNF-α and IFN-γ upon coculture with autologous PBMC containing leukemic B cells but not with PBMC of healthy donors; this production was highly increased when PWM-activated autologous PBMCs were used as stimulators, whereas no secretion was detected in the supernatants of PWM-activated B-CLL cells nor of autologous PHA-activated T cells (Fig. 1*C*). Vδ2 T cells secreted low amounts of TNF-α or IFN-γ, even in the presence of activated autologous PBMCs (Fig. 1*C*).

Activated B-CLL Cells Are Killed by Autologous Vδ1 T Lymphocytes via NKG2D. In 10 of 15 patients with γδ T cell expansion (patients 01, 03, 04, 05, 06, 21, 23, 25, 26, and 28; Table 1) we could obtain a sufficient number of Vδ1 or Vδ2 T cells to be studied in detail. Autologous leukemic B cells were also separated, obtaining a purity of >99% as evaluated by CD20/CD5 staining, and used as target cells in cytotoxicity assay. Each γδ T cell population was recognized by the specific anti-Vδ1 (A13 or MCA 2080) or anti-Vδ2 (BB3 or γδ123R3) mAbs, but not by the anti-αβ–T-cell receptor mAb BAM031. Both Vδ1 and Vδ2 T cell lines were NKG2D positive, the intensity of NKG2D expression of all Vδ2 cell lines analyzed being lower than that of the Vδ1 cell lines (mean fluorescence intensity, 50 ± 8 *versus* 140 ± 10 a.u.; data not shown in tables or figures).

Fig. 1*D* shows that the cytolytic activity exerted by Vδ1 T lymphocytes against autologous neoplastic B cells was low; similar results were obtained using Vδ2 T-cell lines as effectors (data not shown). However, leukemic B cells were efficiently killed by autologous Vδ1 T lymphocytes when activated with a polyclonal mitogen (PWM; Fig. 1*D*); interestingly, this cytotoxicity was inhibited by adding an anti-NKG2D mAb (Fig. 1D), indicating that this molecule is involved in the recognition of the ligands expressed by B-CLL cells. On the other hand, Vδ2 cell lines did not kill efficiently the autologous B cells, even if when activated, whereas they were cytotoxic for the Burkitt lymphoma cell lines Daudi and Raji (data not shown), as described previously (9).

B-CLL Cells Express *De novo* MIC-A and Up-Regulate ULBP3 Upon Treatment with All-*Trans*-Retinoic Acid. Given that NKG2D has been reported to recognize the MHC-related molecules MIC-A and the UL16-binding proteins ULPBs (11, 19, 22, 24), we focused our attention on the expression of these molecules upon B-CLL cell activation. As the expression of the murine MHC-related RAE-1 molecule is up-regulated by retinoic acid (24, 32), we also investigated the effect of ATRA on leukemic B cells. *Ex vivo* B-CLL cells did not express MIC-A, either as mRNA (Fig. 2*A*) or as membrane protein (Fig. 2*B*). After treatment with PWM, transcription of mRNA coding for MIC-A was observed at 24 hours (Fig. 2*A*), whereas the protein surface expression became detectable on B-CLL cells at day 3 (Fig. 2*B*, results are referred to patient 01). Treatment with ATRA induced in B-CLL cells from 4 of the 10 patients studied (patients 01, 03, 04, and 07) the transcription of MIC-A starting from 20 minutes up to 24 hours (Fig. 2*A*, Lanes 2 to 4) and membrane expression at 24 hours (Fig. 2*B*). Kinetics experiments, with B-CLL from patients 01, 03, 04, and 07, showed that the peak of MIC-A expression after PWM stimulation was at day 5, whereas upon treatment with ATRA, the maximal surface expression occurred after 3 days (Fig. 2*C*). The apparent discrepancy between the amount of MIC-A mRNA detectable 1 day after exposure to ATRA and the protein detectable for ≥3 days might be attributable to the relative instability of the mRNA, compared with the turnover and half-life of the protein. No basal detection or induction of mRNA coding for MIC-B was observed (data not shown).

As the cell surface expression of MIC-A was not induced by ATRA in all of the B-CLL studied, and as the UL16-binding proteins ULBPs have also been reported as NKG2D ligands (22, 24), we next investigated the expression of these molecules. Fig. 3*A* shows that ULBP3 mRNA transcription is up-regulated in B-CLL (10 of 10 patients analyzed, one representative patient depicted) early after treatment with ATRA or PWM. Densitometric analysis showed a 2-fold increase in ULBP3 mRNA 20 minutes after exposure to ATRA (Fig. 3*A*, Lane 2 *versus* Lane 1), reaching a pick with a 3-fold increase at 40 minutes (Fig. 3*A*, Lane 3 *versus* Lane 1) and being detectable also after overnight incubation with either ATRA (Fig. 3*A*, Lane 4 *versus*

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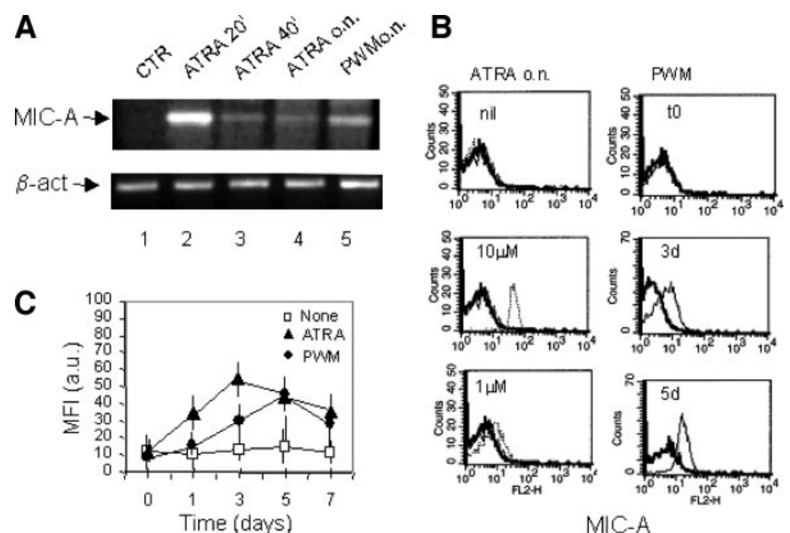

Fig. 2. Induction of MIC-A on leukemic B cells by ATRA and PWM. *A*, MIC-A mRNA expression evaluated in B-CLL (1 representative sample of 10 analyzed), untreated or exposed to ATRA for 20 or 40 minutes, or 24 hours (o.n., overnight) or to PWM for 24 hours (o.n.) as indicated by PCR as described in Materials and Methods. *B*, expression of MIC-A at the cell surface of B cells, untreated or exposed to ATRA (10 or 1 μmol/L) for 24 hours or to PWM (100 ng/mL) for 3 days or 5 days, as indicated, evaluated by indirect immunofluorescence with the specific anti-MIC-A mAb. Samples were analyzed on a flow cytometer (FACScan) equipped with an argon ion laser. Data are expressed as mean fluorescence intensity (MFI, a.u., *X* axis) *versus* number of cells (*Y* axis). Data in this panel are referred to one patient (03) of four patients (01, 03, 04, and 07). *C*, kinetics of expression of MIC-A at the cell surface of B cells, untreated (none) or exposed to ATRA (10 μmol/L) or to PWM (100 ng/mL) for the indicated time points, evaluated by indirect immunofluorescence with the specific anti-MIC-A mAb. Samples were analyzed on a FACSort; results depicted in this panel are referred to four different patients (01, 03, 04, and 07) and SD of MFI is indicated at each time point.

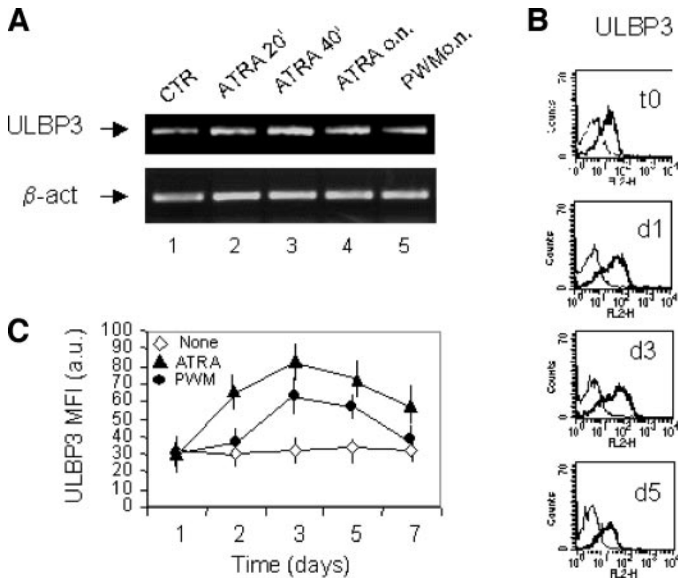

Fig. 3. Induction of ULBP3 on leukemic B cells by ATRA or PWM. *A*, ULBP3 mRNA expression evaluated in B-CLL, untreated or exposed to ATRA for 20 minutes, 40 minutes, or 24 hours (o.n., overnight) or to PWM for 24 hours (o.n.), as indicated by PCR as described in Materials and Methods (patient 01 is depicted). *B*, expression of ULBP3 at the cell surface of B cells, untreated or exposed to ATRA (10 μmol/L) for the indicated periods of time, evaluated by indirect immunofluorescence. Samples were analyzed on a FACSort; data are expressed as mean fluorescence intensity (a.u., *X* axis) *versus* number of cells (*Y* axis). *C*, kinetics of expression of ULBP3 at the cell surface of B cells, untreated (none) or exposed to ATRA (10 μmol/L) or to PWM (100 ng/mL) for the indicated time points, evaluated by indirect immunofluorescence with the specific anti-ULBP3 mAb. Samples were analyzed on a FACSort, are expressed as MFI (a.u.), and are the mean ± SD from 10 patients.

Lane 1) or PWN (Fig. 3*A*, Lane 5 *versus* Lane 1: 2-fold increase). Similar results were obtained with all of the 10 patients analyzed. No transcripts for ULBP1, ULBP2, and ULBP4 could be amplified (data not shown). ULBP3 could be found, at low intensity, on the cell surface of *ex vivo* isolated B-CLL cells (Fig. 3*B*: mean fluorescence intensity, 36 ± 4 a.u.; negative control, 10 ± 4 a.u.), and it was strongly up-regulated upon exposure to PWM (mean fluorescence intensity, 78 ± 5 a.u.) or ATRA (mean fluorescence intensity, 87 ± 6 a.u.; one representative patient of 10). Kinetics experiments showed that ULBP3 expression was maximal after 2 to 3 days for ATRA-treated B cells and after 4 to 5 days for PWM-treated B cells (Fig. 3*C*; mean ± SD from 10 patients). On the contrary, ULBP1, ULBP2, and ULBP4 molecules were not expressed on the cell surface either before or after treatment of B-CLL cells with either ATRA or PWM (data not shown).

Vδ1 T Lymphocytes Kill ULBP3-positive Autologous B-CLL Cells. Additional evidence for the ability of Vδ1 T lymphocytes from B-CLL patients to kill tumor B cells due to the expression of NKG2D ligands came from a series of experiments performed with the $ULBP3^+$ C1R lymphoma cell line, untransfected or stably transfected with MIC-A. Interestingly, Vδ1 T-cell lines obtained from the patients studied were able to lyse MIC-A–positive C1R targets, with a much higher efficiency than the wild-type untransfected cell line (Fig. 4*A*). Of note, the cytolytic activity exerted by Vδ1 T-cell lines against MIC-A/C1R targets could be inhibited by blocking NKG2D (Fig. 4*A*) on the effector cells or MIC-A and/or ULBP3 on target cells, with the $F(ab')_2$ of the specific mAbs (Fig. 4*A*), suggesting that tumor cell lysis is dependent on MIC-A/ULBP3 interaction with their receptor(s). Conversely, covering HLA-I molecules did not inhibit tumor cell lysis (Fig. 4*A*). More importantly, autologous leukemic B cells exposed to either PWM or ATRA became susceptible to lysis by Vδ1 T-cell lines (Fig. 4*B*); again, the addition of either anti-NKG2D or anti-ULBP3 mAbs strongly inhibited the Vδ1-mediated lysis of leukemic B cells, whereas the anti-HLA-I mAb did not (Fig. 4*B*). The finding that blocking of one ligand but not of the other leads to an almost complete inhibition of NKG2D-mediated lysis of ULBP3-positive targets might be due to a threshold effect, *i.e.,* once the receptor is activated by one ligand the signal transduction occurs as an all-or-none mode of action. Conversely, Vδ2 T-cell lines displayed only a slight cytolytic activity against MIC-A–transfected lymphoma cells, as well as against either ATRA or PWM-treated autologous B-CLL (data not shown).

Analysis of ULBP3 Expression, Peripheral Vδ1 Count, and 1-Year Follow-up of B-CLL Patients. Interestingly, in 6 of the 17 low-risk/intermediate-risk patients (one third) who had a low number of circulating Vδ1 T cells the disease progressed over 1 year (2 patients from low risk to intermediate risk and 4 from intermediate risk to high risk); these patients displayed poor prognostic markers, such as the presence of CD38 on B-CLL cells and unmutated IgV_H genes, and ULBP3 was not expressed nor inducible at the surface of

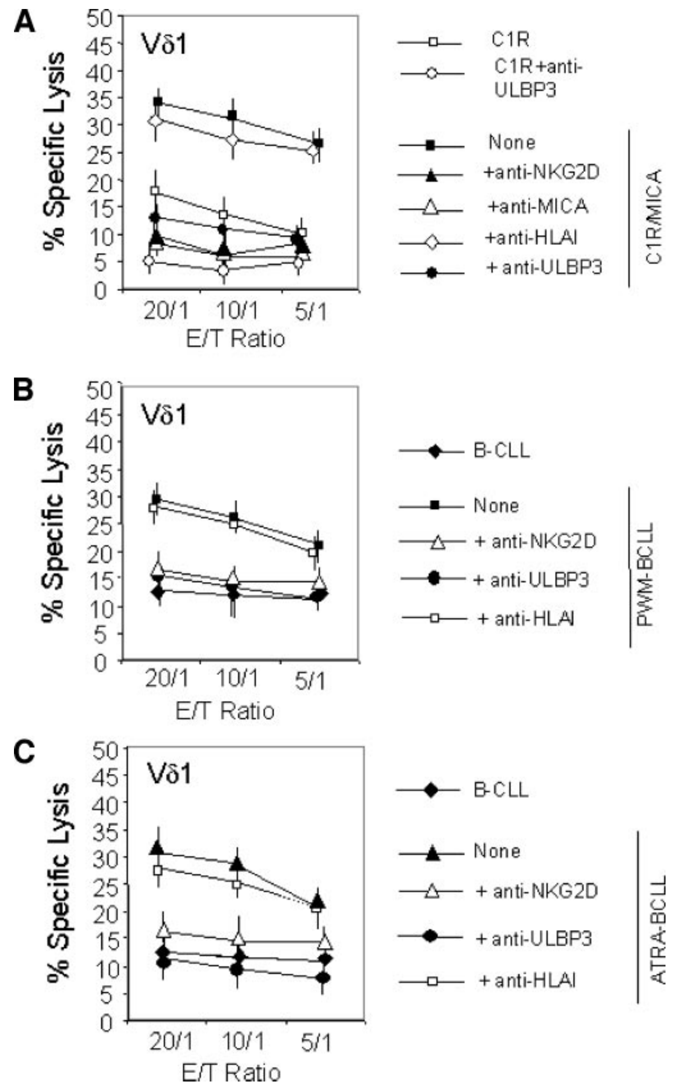

Fig. 4. MIC-A or ULBP3 positive B-CLL cells are killed by Vδ1 T lymphocytes. Vδ1 T-cell lines obtained from 10 B-CLL patients were tested in 4-hour ^{51}Cr release assay against the C1R lymphoma cell line, untransfected or stably transfected with MIC-A (*A*) or against autologous B-CLL cells, resting or activated for 24 hours with PWM (10 μg/mL; *B*) or ATRA (10 μmol/L; *C*), at the indicated E:T ratios. In some experiments, covering of NKG2D on effector cells or of MIC-A or ULBP3 on target cells was performed with the specific mAbs at the concentration of 5 μg/mL. The anti-HLA-I mAb was used, at 5 μg/mL, as a control. None: no mAb added. Results are expressed as percentage specific lysis as described in Materials and Methods and are the mean ± SD from experiments performed with cell lines obtained from 10 patients.

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their leukemic B-CLL cells (Table 2). Likewise, all of the high-risk patients had $CD38^{+}$ B-CLL cells, unmutated IgV_H genes, low Vδ1 T-cell number, and low or undetectable ULPB3 expression, and all but one ULPB3 was not inducible (Table 2). At variance, patients with high Vδ1 T lymphocyte counts showed stable disease, their B-CLL cells were $CD38^{-}$, IgV_H genes were mutated and ULBP3 was expressed and up-regulated on the surface of leukemic B cells (Table 2).

All of the results reported above, suggest a possible a role of Vδ1 T lymphocytes in the defense against B-cell leukemias. This activity might not take place when the interaction between NKG2D and MIC-A or ULBPs is impaired by the absence or low expression of these NKG2D ligands on the cell surface of leukemic B cells. It has been recently reported that such interaction might also be abolished by soluble MIC-A or related molecules released by tumor cells (33, 34). Indeed, we could find detectable amounts of soluble MIC-A in the supernatant of *ex vivo* isolated leukemic B cells, obtained from 10 B-CLL patients, and cultured with PWM for 48 hours (>2.5 ng/mL; range, 1.8 to 3.5 ng/mL); conversely, autologous T cells stimulated with PHA did not release soluble MIC-A (<0.2 ng/mL; data not shown). Of note, ATRA could induce transcription and expression of MIC-A in four cases but did not increase the release of the soluble molecule in none of the 10 cases analyzed (<0.4 ng/mL; range, 0 to 0.6 ng/mL). Soluble MIC-A was also detected (1 to 3.5 ng/mL) in the sera of 6 of 38 B-CLL patients (Table 2), suggesting that release of MIC-A may also occur *in vivo* in some patients, thus possibly influencing the development of antitumor reactivity. Interestingly, all patients with detectable soluble MIC-A had low numbers of circulating Vδ1 T cells and showed a disease progression over 1 year (Table 2). Moreover, four of five high-risk patients with high levels of soluble MICA in the serum (>2.0 ng/mL; Table 2) showed very low or undetectable expression of NKG2D on peripheral blood lymphocytes (mean fluorescence intensity < 10 a.u.), whereas the expression of NKG2D in all low-risk patients with undetectable soluble MICA was comparable with that of normal resting cells (mean fluorescence intensity, 65 ± 23 a.u.; data not shown in figures or tables).

DISCUSSION

In this work, we have investigated the possibility that the Vδ1 γδ T-cell subset may play a role in the antitumor response against B-CLL. This possibility is based on a number of considerations. An increase in circulating Vδ1 T lymphocytes, usually belonging to the resident, mucosal associated cell population, has been reported in acute myeloid leukemias (13). Their presence has been taken to suggest that Vδ1 γδ T cells may either exert an antitumor effect by themselves or can be used to potentiate antitumor therapies. However, a direct antileukemic effect of this γδ T-cell subset has not been reported thus far.

We here provide a 3-fold evidence that Vδ1 γδT-cell subset may be involved also in the antitumor response against B-CLL. First, in about half (15 of 38) B-CLL patients, mostly at low-risk stage, circulating Vδ1 T lymphocytes were significantly increased as compared with healthy donors matched for sex and age, whereas no increase in Vδ1 γδT cells was found in most of intermediate-risk patients and in all six patients with advanced disease (high risk). In 13 of the 15 patients with high Vδ1 count, neoplastic B cells were $CD38^{-}$, and in 7 patients, the IgV_H genes were mutated. Of note, in 6 of the 17 patients (one third) who did not have an increased number of circulating Vδ1 T cells, a disease progression has been observed over 1 year. In all these patients and in the majority of the patients with low count of peripheral Vδ1 T cells, B-CLL cells were $CD38^{+}$ and IgV_H genes were unmutated, *i.e.,* they had markers of negative prognostic value (29–31, 35).

Second, Vδ1 T cells obtained from B-CLL patients proliferate in response to autologous leukemic B cells and produce TNF-α or IFN-γ, in keeping with a recent report showing that γδ T cells can provide an early source of antineoplastic cytokines in a murine model (36). That notwithstanding they are unable to efficiently kill autologous leukemic B cells as well as lymphoma cell lines. This inability might be attributable to the lack, or low expression, of the stress-inducible, MHC-related MIC-A molecule, or of the UL16-

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Table 2 *Characteristics of B-CLL patients: follow up at 1 year*

Patients *	Stage at diagnosis †	Stage in 2004 ‡	Surface CD38 §	IgG V/H ¶	Vδ1 (cells/μL^{-1}) in 2003 ‖	Vδ1 (cells/μL) in 2004 **	Constitutive ULBP3 ††	Induced ULBP3 ‡‡	sMICA (ng/mL) §§
11	IR	HR	$CD38^{+}$	M	23	29	5	15	2.0
13	IR	HR	$CD38^{+}$	ND	27	21	16	20	1.8
19	IR	HR	$CD38^{+}$	U	12	15	18	49	3.6
22	LR	IR	$CD38^{+}$	U	36	30	15	14	0.9
30	LR	IR	$CD38^{+}$	U	4	10	12	15	3.0
32	IR	HR	$CD38^{+}$	U	3	9	22	18	2.4
33	HR	HR	$CD38^{+}$	U	21	12	12	10	1.0
36	HR	HR	$CD38^{+}$	U	6	7	11	10	0.5
37	HR	HR	$CD38^{+}$	U	10	11	11	28	2.0
01	LR	LR	$CD38^{-}$	M	366	340	44	86	0.0
03	LR	LR	$CD38^{-}$	M	389	400	33	58	0.1
04	LR	LR	$CD38^{-}$	M	276	290	31	77	0.1
12	LR	LR	$CD38^{-}$	M	149	162	33	87	0.2
21	LR	LR	$CD38^{-}$	M	240	234	42	98	0.0
25	LR	LR	$CD38^{-}$	M	280	260	36	85	0.2
26	LR	LR	$CD38^{-}$	M	187	210	52	99	0.0
28	LR	LR	$CD38^{-}$	M	238	255	32	75	0.1

* Chemonaive patients who never had received chemotherapy.
† Stage at diagnosis defined according to the Rai modified classification (26): LR, low risk; IR, intermediate risk; HR, high risk.
‡ Stage in 2004 defined according to the Rai modified classification (26).
§ Percentage of $CD38^{+}$ leukemic B cells is reported in Table 1.
¶ IgG mutation were evaluated as described previously (29). The sequences with a germ-line homology ≥ 98% were considered unmutated (U) and those with a homology < 98% mutated (M) (31, 35). ND, not done.
‖ Absolute number of peripheral blood Vδ1 T lymphocytes/μL at the beginning of the study (early 2003).
** Absolute number of peripheral blood Vδ1 T lymphocytes/μL after 1 year (2004).
†† ULBP3 surface expression evaluated by immunofluorescence and cytofluorimetric analysis with the specific anti-ULBP mAb labeled with Alexafluor594; results are expressed as mean fluorescence intensity (a.u.); mean fluorescence intensity of Alexafluor-labeled, isotype-matched irrelevant mAb was 15 a.u. These data were confirmed by PCR.
‡‡ ULBP3 surface expression evaluated by immunofluorescence and cytofluorimetric analysis as above, after exposure to ATRA (10 μg/mL) for 5 days. These data were confirmed by PCR.
§§ Soluble MIC-A measured by ELISA in the sera of B-CLL patients in 2003; results are expressed as ng/mL.

binding proteins ULBPs, all reported ligands for NKG2D (19, 21, 22, 24).

However, in a fraction of B-CLL cases a low but detectable expression of ULBP3 was found: the low expression of this molecule might be sufficient to induce Vδ1 T-cell proliferation but not cytotoxicity. We propose that the role of Vδ1 T lymphocytes, which usually represent a resident lymphocyte population, is played at the tumor site, such as bone marrow or lymph nodes in the case of B-CLL. In these sites, a transient but efficient expression of NKG2D ligands might occur because of the production of soluble factors with effects possibly superimposable to those mimicked *in vitro* by activating agents such as PWM. Along this line, preliminary histochemical analyses on bone marrow or lymph node specimens from two CLL patients provide evidence for the expression of ULBP3 on neoplastic B lymphocytes (data not shown). Thus, the increased levels of circulating Vδ1 T cells would be a consequence of a response that had taken place in affected tissues.

The third piece of evidence is that Vδ1 T-cell populations isolated from B-CLL patients not only can kill neoplastic B-cell lines transfected with MIC-A but also acquire the capability of killing autologous leukemic cells through NKG2D, provided that the expression of its ligands, *i.e.,* MIC-A or ULBP3, is induced or up-regulated on the surface of leukemic B cells upon activation with PWM or exposure to ATRA. The UL16-binding proteins ULBPs, as MIC-A, are expressed by several tumor cell lines and in some acute leukemias (20, 28). In mice, a family of proteins structurally related to ULBPs, the RAE-1 molecules, bind to NKG2D and induce cell activation (24, 37). Moreover, RAE-1 expression has been shown to be induced by carcinogens and stimulate antitumor activity of $\gamma\delta$ lymphocytes (19). It is of note that B-CLL cells can up-regulate mRNA transcripts and expression of ULPB3 upon polyclonal activation or exposure to ATRA, leading to recognition by autologous Vδ1 T cells, via NKG2D activation, and tumor cell killing. Circulating B-CLL cells, which are in a kinetically resting state, may lack, or express at low levels, the relevant antigens recognized by $\gamma\delta$ T lymphocytes, and this possibly prevents or impairs their elimination. More importantly, we found that in most low-risk patients the expression of ULBP3 on the surface of leukemic cells and an increased number of circulating Vδ1 T lymphocytes correlate with favorable prognostic factors, such as the absence of CD38 and mutated IgV_H genes and is associated with disease stability. On the contrary, in patients with low counts of Vδ1 T lymphocytes and $ULBP^-$ B-CLL cells, the malignant cells also present unfavorable prognostic factors such as the presence of CD38 or unmutated IgVH genes and the disease progressed over 1 year.

Recently, it has been demonstrated that MIC-A is released as a soluble form from the surface of tumor cells and can be detected in the sera of patients with gastroenteric malignancies and acute myeloid leukemias (33, 34). Our findings that, in some cases, cultured activated leukemic B cells can release soluble MIC-A in the supernatant suggest that Vδ1 lymphocytes in B-CLL patients may be inhibited in their function because of the occupancy of NKG2D by the soluble molecule before $\gamma\delta$ T cells encounter the tumor target. Soluble MIC-A could also be detected in the sera of six of these patients, all with low Vδ1 count in peripheral blood and showing poor prognostic markers. Along this line, the expression of NKG2D in low-risk patients with undetectable soluble MICA was comparable with that of normal resting cells, whereas three high-risk patients with high levels of soluble MICA in the serum showed very low or undetectable expression of NKG2D on peripheral blood lymphocytes. An *in vitro* assay for the determination of soluble ULBPs is not yet available; these molecules, structurally related to MIC-A, might be much more expressed on the cell surface in hematologic malignancies than MIC-A and MIC-B; however, once released, they may also represent a mechanism of tumor escape from anticancer surveillance. On the contrary, enhanced surface expression, induced by drugs such as ATRA, in the absence of increased release of the soluble forms might rescue tumor susceptibility to Vδ1-mediated cytotoxic activity.

In conclusion, our results point to a possible role for Vδ1 T lymphocyte in the antitumor defense against B-CLL, highlight a potential therapeutic use of retinoic acid in enhancing the activation of the $\gamma\delta$ T-cell subset, which preferentially responds to B-CLL tumor cells and lead to suggest that, as proposed for other hematologic malignancies (28), determination of soluble MIC-A might be of value in the follow up of B-CLL patients.

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