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

Alessandro Poggi,1 Claudia Venturini,2 Silvia Catellani,2 Marino Clavio,2 Maurizio Migliore,2 Marco Gobbi,2 Alexander Steinle,3 Paolo Ghia,4 Stefania Stella,4 Federico Caligaris-Cappio,4 and Maria Raffaella Zocchi2,5

1Laboratory of Immunology, National Cancer Research Institute, Genoa, Italy; 2Clinical Hematology, Department of Internal Medicine, University of Genoa, Genoa, Italy; 3Department of Immunology, Eberhard-Karls University, Tuebingen, Germany; 4Laboratory of Immunology and Oncology, Institute for Cancer Research, Candiolo, Italy; and 5Laboratory of Tumor Immunology and Department of Oncology, Università Vita Salute and Istituto di Ricovero e Cura a Carattere Scientifico San Raffaele, Milan, Italy

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 to 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 MICA 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+ CIR B-cell line upon transfection with MICA. More importantly, they also lysed autologous B-CLL cells when transcription and expression of MICA 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 recognizes 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 yδ123R3 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 AM01 and BAM03 were from Immatics Biotechnologies (Tubingen, Germany), and the anti-ULPB mAbs (anti-ULBP1 M291, anti-ULBP2 M311, anti-ULBP3 M551, anti-ULBP4 M478) were...
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 36 T-Cell Populations. Peripheral blood mononuclear cells (PBMCs) from B-CLL patients and healthy donors were isolated by Ficoll-Hypaque gradient. Highly purified CD3-negative T lymphocytes were obtained from isolated PBMCs as feeder cells as described previously (27). V1R1 or V1R2 T lymphocytes/µL were added to the cultures seeded under limiting dilution conditions in 96-well U-bottomed microwells (Greiner Labortecnic, Nurtingen, Germany) and cultured in RPMI 1640 supplemented with 200 µmol/L L-glutamine 10% of FCS, 1 µg/mL PHA, 25 units/µL recombinant interleukin 2, and 10,000 irradiated (3000 rad) autologous PBMCs as feeder cells as described previously (27). V61 or V62 T-cell lines, CD4-C8 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 V61 or V62 T-cell lines, CD4-C8 cells were seeded under limiting dilution conditions in 96-well U-bottomed microwells (Greiner Labortecnic, Nurtingen, Germany) and cultured in RPMI 1640 supplemented with 200 µmol/L L-glutamine 10% of FCS, 1 µg/mL PHA, 25 units/µL recombinant interleukin 2, and 10,000 irradiated (3000 rad) autologous PBMCs as feeder cells as described previously (27). V61 cell lines were A13 MCA20805, V62 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 V61 or V62 T cells to autologous B-CLL PBMCs versus normal irradiated resting or PWM-activated PBMCs was evaluated by counting the number of A13 MCA20805 (V61) or BBS5 (V62) 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, 10, and 15 units/µL recombinant interleukin 2 were added to the cultures.

Immunofluorescence and Cytometric 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 software (Becton Dickinson) equipped with an argon ion laser exciting phycoerythrin at 488 nm. Calibration was assessed with CALIBRITE particles (Becton Dickinson) with the AutoCOMP computer program.

Cytotoxicity Assay. Cytolytic activity of V61 or V62 T-cell lines from B-CLL patients was analyzed in a 4-hour 51Cr-release assay against the B lymphoma cell lines Daadi and Raji or against the autologous or allogeneic tumor B cells, labeled with 51Cr, at an E:T ratio of 40:1 to 1:1, 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 51Cr-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
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 MICA, MIC-B, and ULBPs. Total RNA was prepared from PBMCs with TRIPure (Sigma Chemicals Co.) and reverse transcribed with the RT kit from Ampliterm (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: \( 5'\)-CATTACTCTGTCTGTGATCC-3' and \( 5'\)-ACCTCACCATGAAAGGTGTTGACG-3'.

RESULTS

\[ 3'\]CH primer. The PCR products were sequenced with the appropriate antisense CH 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 described previously (29). After determining the IgVH gene family used by the leukemic cells, the VH gene sequences were determined by amplifying 2.5 mmol reaction mixtures containing 10 U Taq DNA polymerase (PeproTec).

DETECTION OF \( \text{IgV}_{\text{H}} \) GENES

In 24 of 38 patients the analysis of \( \text{IgV}_{\text{H}} \) genes mutational status was performed on cDNA as described previously (29). After determining the \( \text{IgV}_{\text{H}} \) gene family used by the leukemic cells, the \( \text{IgV}_{\text{H}} \) gene sequences were determined by amplifying 2.5 \( \mu \)L of cDNA by PCR with the appropriate sense \( \text{IgV}_{\text{H}} \) leader primer in combination with the appropriate antisense \( \text{CH} \) 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). 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 MICA, TNF-\( \alpha \), and IFN-\( \gamma \). Soluble MICA 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-MICA mAb AMO-1 at 5 \( \mu \)g/mL in PBS overnight at 4°C and blocked with 7% PBS-BSA for 2 hours at 37°C. Standard (recombinant MICA-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 anti-MICA mAb BAMO3 was added at 2 \( \mu \)g/mL for 2 hours at 37°C, followed by the horseradish peroxidase-conjugated antimonkey IgG2a antiserum (Sigma Chemicals Co.) at 1:10,000 dilution for 1 hour at 37°C. Plated were then developed with the appropriate mAbs before start the cytotoxicity assay. Statistical analysis was performed with the Student t test.

Evaluation of mRNA for MICA, MIC-B, and ULBPs. Total RNA was prepared from PBMCs with TRIPure (Sigma Chemicals Co.) and reverse transcribed with the RT kit from Ampliterm (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: \( 5'\)-CATTACTCTGTCTGTGATCC-3' and \( 5'\)-ACCTCACCATGAAAGGTGTTGACG-3'.

RESULTS

V\( \text{V}_{\text{1}} \) T Lymphocytes from B-CLL Patients with Stable Disease

Are Increased in Peripheral Blood, Proliferate, and Produce TNF-\( \alpha \) and IFN-\( \gamma \) 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 V\( \text{V}_{\text{1}} \) T lymphocytes, which usually belong to the resident population of V\( \text{V}_{\text{1}} \) T cells and are barely detectable in peripheral blood, were significantly increased (mean value, 150; range, 100 to 300 cells/\( \mu \)L; Table 1) compared with 15 healthy donors matched for sex and age (mean value, 54; range, 50 to 100 cells/\( \mu \)L; \( P < 0.05 \); 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 \( \text{IgV}_{\text{H}} \) genes were mutated (Table 1). In the same patients, V\( \text{V}_{\text{2}} \) T cells were only slightly increased (120 to 200 versus 100 to 150 cells per \( \mu \)L of healthy donors), not reaching statistical significance (data not shown). The remaining 23 patients had a normal or low V\( \text{V}_{\text{1}} \) count (12 low risk, 5 intermediate risk, and 6 high risk), their B cells were mostly CD38\(^{+}\) (17 of 23), and Ig\( \text{V}_{\text{H}} \) genes were unmutated in more than half (13 of 23) of the patients (Table 1). More specifically, five of the high-risk patients were CD38\(^{+}\), four were unmethylated, and none had increased V\( \text{V}_{\text{1}} \) T cells (Table 1).

When purified, V\( \text{V}_{\text{1}} \) T lymphocytes were cultured in the presence of irradiated autologous B-CLL PBMCs as feeder cells, a strong proliferation of the V\( \text{V}_{\text{1}} \) 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. 1A). This effect was more evident (100-fold increase) when PWM-activated autologous PBMCs were used as stimulants.

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ululators; on the contrary, no evident proliferation occurred when V61 T cells were cultured in the presence of PBMCs of healthy donors, irrespective of their activation (Fig. 1A). As for V92 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 V61 T lymphocytes (Fig. 1B versus Fig. 1A).

These data suggest that in B-CLL patients the expansion of V61 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 V61 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. 1A). Importantly, we found that γδ T cells from B-CLL patients can produce cytokines with potential antitumor activity: in particular, V61 γδ 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. 1C). V62 T cells secreted low amounts of TNF-α or IFN-γ, even in the presence of activated autologous PBMCs (Fig. 1C).

### Activated B-CLL Cells Are Killed by Autologous V61 T Lymphocytes via NKG2D.

In 10 of 15 patients with γδ V61 T cell expansion (patients 01, 03, 04, 05, 06, 21, 23, 25, 26, and 28; Table 1) we could obtain a sufficient number of V61 or V62 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-V61 (A13 or MCA 2080) or anti-V92 (BB3 or y8123R3) mAbs, but not by the anti-αβ T–cell receptor mAb BAM031. Both V61 and V62 T cell lines were NKG2D positive, the intensity of NKG2D expression of all V62 cell lines analyzed being lower than that of the V61 cell lines (mean fluorescence intensity, 50 ± 8 versus 140 ± 10 a.u.; data not shown in tables or figures).

Fig. 1D shows that the cytolytic activity exerted by V61 T lymphocytes against autologous neoplastic B cells was low; similar results were obtained using V92 T-cell lines as effectors (data not shown). However, leukemic B cells were efficiently killed by autologous V61 T lymphocytes when activated with a polyclonal mitogen (PWM; Fig. 1D); 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, V62 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 ULBPbs (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. 2A) or as membrane protein (Fig. 2B). After treatment with PWM, transcription of mRNA coding for MIC-A was observed at 24 hours (Fig. 2A), whereas the protein surface expression became detectable on B-CLL cells at day 3 (Fig. 2B, 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. 2A, Lanes 2 to 4) and membrane expression at 24 hours (Fig. 2B). 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. 2C). 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 ULBPbs have also been reported as NKG2D ligands (22, 24), we next investigated the expression of these molecules. Fig. 3A 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. 3A, Lane 2 versus Lane 1), reaching a peak with a 3-fold increase at 40 minutes (Fig. 3A, Lane 3 versus Lane 1) and being detectable also after overnight incubation with either ATRA (Fig. 3A, Lane 4 versus...
at the cell surface of B cells, untreated or exposed to ATRA (10 μM) 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 μM) for the indicated periods of time, as 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 μM) or to PWM (100 ng/mL) for the indicated time points, as evaluated by indirect immunofluorescence with the specific anti-ULBP3 mAb. Samples were analyzed on a FACSort, as expressed as MFI (a.u.), and are the mean ± SD from 10 patients.

Lane 1) or PWM (Fig. 3A, 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. 3B: 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. 3C; 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).

V61 T Lymphocytes Kill ULBP3-positive Autologous B-CLL Cells. Additional evidence for the ability of V61 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+/CIR lymphoma cell line, untransfected or stably transfected with MIC-A. Interestingly, V61 T-cell lines obtained from the patients studied were able to lyse MIC-A-positive CIR targets, with a much higher efficiency than the wild-type untransfected cell line (Fig. 4A). Of note, the cytolytic activity exerted by V61 T-cell lines against MIC-A/CIR targets could be inhibited by blocking NKG2D (Fig. 4A) on the effector cells or against ULBP3 on target cells, with the F(ab′)2 of the specific mAbs (Fig. 4A), 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. 4A). More importantly, autologous leukemic B cells exposed to either PWM or ATRA became susceptible to lysis by V61 T-cell lines (Fig. 4B); again, the addition of either anti-NKG2D or anti-ULBP3 mAbs strongly inhibited the V61-mediated lysis of leukemic B cells, whereas the anti-HLA-I mAb did not (Fig. 4B). 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, V62 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 V61 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 V61 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 unmaturated IgVH genes, and ULBP3 was not expressed nor inducible at the surface of
their leukemic B-CLL cells (Table 2). Likewise, all of the high-risk patients had CD38+ B-CLL cells, unmaturated IgVH genes, low V61 T-cell number, and low or undetectable ULPB3 expression, and all but one ULPB3 was not inducible (Table 2). At variance, patients with high V61 T lymphocyte counts showed stable disease, their B-CLL cells were CD38-, IgVH genes were mutated and ULPB3 was expressed and up-regulated on the surface of leukemic B cells (Table 2).

All of the results reported above, suggest a possible role of V61 T lymphocytes in the defense against B-cell leukemias. This activity might not take place when the interaction between NKGD2 and MIC-A or ULPBs is impaired by the absence or low expression of these NKGD2 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 detect 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 V61 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 NKGD2 on peripheral blood lymphocytes (mean fluorescence intensity <10 a.u.), whereas the expression of NKGD2 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 V61 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 V61 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 V61 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 V61 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 V61 T lymphocytes were significantly increased as compared with healthy donors matched for sex and age, whereas no increase in V61 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 V61 count, neoplastic B cells were CD38+, and in 7 patients, the IgVH genes were mutated. Of note, in 6 of the 17 patients (one third) who did not have an increased number of circulating V61 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 V61 T cells, B-CLL cells were CD38- and IgVH genes were unmaturated, i.e., they had markers of negative prognostic value (29–31, 35).

Second, V61 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-
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 γδ T lymphocytes (19). It is of note that B-CLL cells can up-regulate mRNA transcripts and expression of ULBP3 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 Vδ1 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 IgVδ 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 γδ 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 lymphocytes in the antitumor defense against B-CLL, highlight a potential therapeutic use of retinoic acid in enhancing the activation of the γδ 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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V<sup>δ1</sup> T Lymphocytes from B-CLL Patients Recognize ULBP3 Expressed on Leukemic B Cells and Up-Regulated by Trans-Retinoic Acid

Alessandro Poggi, Claudia Venturino, Silvia Catellani, et al.


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