The Journal of Immunology

RESEARCH ARTICLE | MARCH 15 2010

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J Immunol (2010) 184 (6): 3260–3268.
https://doi.org/10.4049/jimmunol.0903454

Vγ9Vδ2 T Lymphocytes Efficiently Recognize and Kill Zoledronate-Sensitized, Imatinib-Sensitive, and Imatinib-Resistant Chronic Myelogenous Leukemia Cells

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Imatinib mesylate (imatinib), a competitive inhibitor of the BCR-ABL tyrosine kinase, is highly effective against chronic myelogenous leukemia (CML) cells. However, because 20–30% of patients affected by CML display either primary or secondary resistance to imatinib, intentional activation of Vγ9Vδ2 T cells by phosphoantigens or by agents that cause their accumulation within cells, such as zoledronate, may represent a promising strategy for the design of a novel and highly innovative immunotherapy capable to overcome imatinib resistance. In this study, we show that Vγ9Vδ2 T lymphocytes recognize, trogocytose, and efficiently kill imatinib-sensitive and -resistant CML cell lines pretreated with zoledronate. Vγ9Vδ2 T cell cytotoxicity was largely dependent on the granule exocytosis- and partly on TRAIL-mediated pathways, was TCR-mediated, and required isoprenoid biosynthesis by zoledronate-treated CML cells. Importantly, Vγ9Vδ2 T cells from patients with CML can be induced by zoledronate to develop antitumor activity against autologous and allogeneic zoledronate-treated leukemia cells, both in vitro and when transferred into immunodeficient mice in vivo. We conclude that intentional activation of Vγ9Vδ2 T cells by zoledronate may substantially increase their antileukemia activities and represent a novel strategy for CML immunotherapy. ***The Journal of Immunology*, 2010, 184: 3260–3268.**

Chronic myelogenous leukemia (CML) is a malignant hemopoietic stem cell disorder characterized by the reciprocal translocation between chromosomes 9 and 22, resulting in the BCR-ABL oncoprotein (1). Imatinib mesylate (imatinib), a competitive inhibitor of the BCR-ABL tyrosine kinase (TK), is highly effective in CML (2, 3), but frequent relapse has been reported, particularly in patients with advanced-stage disease (4). Although rescue TK inhibitors like desatinib or nilotinib are the preferred choice in this setting, there are patients who are resistant to all TK inhibitors. To overcome this problem, adjunct immunotherapy may provide an opportunity to improve the clinical outcome.

γδ T cells exhibit potent MHC-unrestricted lytic activity against several tumor cells. γδ T cell-based immunotherapy against different tumors has been reported (5–8), and the efficacy of this treatment has been demonstrated both in vivo and in vitro (9–12). The majority of γδ T cells in peripheral blood expressed the variable chain Vδ2 in association with Vγ9 and recognize nonpeptide phosphoantigens commonly associated with metabolites of bacterial isoprenoid biosynthesis or mevalonate pathway in eukaryotes. These compounds, such as isopentenyl pyrophosphate (IPP) and the synthetic analog bromohydrin pyrophosphate (BrHPP), activate γδ T cells in vitro inducing their cytotoxic activity against tumor or infected target cells. Vγ9Vδ2 T cells also respond to nitrogen-containing bisphosphonates (N-BPs), such as zoledronate, probably via accumulation of mevalonate pathway intermediates inside N-BP–treated cells (13).

Recently, interest has emerged on the use of zoledronate in CML, because this drug synergistically augments the anti-Ph$^+$ leukemia activity of imatinib both in vitro and in vivo (14, 15) and inhibits proliferation and induces apoptosis of imatinib-resistant CML cells (16). Moreover, previous studies have demonstrated that zoledronate sensitizes chemotherapy-resistant tumor target cells to Vγ9Vδ2 T cell cytotoxicity, rendering a variety of cancer cell lines highly susceptible to Vγ9Vδ2 T cell-mediated killing (17). Therefore, we reasoned that the intentional activation of Vγ9Vδ2 T cells by zoledronate may represent a promising target for the design of novel and highly innovative immunotherapies capable to overcome imatinib resistance in patients with CML.

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Received for publication October 22, 2009. Accepted for publication December 29, 2009.

This work was supported by grants from the Ministry of University and Research, the Ministry of Health, and the University of Palermo (to G.S. and F.D.). J.J.F. was supported by grants from Institut National de la Santè et de la Recherche Mèdicale, Institut National contre le Cancer, and Association pour la Recherche sur le Cancer. P.V., F.D., and A.M. were supported by grants from Regional and National Associazione Italiana per la Ricerca sul Cancro and the University of Catania. V.O. and M.S. are Ph.D. students of the International Ph.D. Programme in Immunopharmacology at the University of Palermo.

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Abbreviations used in this paper: BrHPP, bromohydrin pyrophosphate; CM, central memory; CMA, concanamycin A; CML, chronic myelogenous leukemia; CMTMR, 5-and-6-(4-chloromethyl-benzoyl-amino-tetramethylrhodamine); EM, effector memory; EMRA, terminally differentiated effector memory; FasL, Fas Ligand; HD, healthy donor; imatinib, imatinib mesylate; IPP, isopentenyl pyrophosphate; MFI, mean fluorescence intensity; NA, viable non apoptotic; N-BP, nitrogen-containing bisphosphonate; Nod, nucleotide-binding oligomerization domain; NVA, nonviable apoptotic; NVNA, nonviable nonapoptotic cell; PI, propidium iodide; TK, tyrosine kinase; VA, viable apoptotic.

www.jimmunol.org/cgi/doi/10.4049/jimmunol.0903454

Promisingly, we show in this study that Vγ9Vδ2 T lymphocytes recognize, trogocytose, and efficiently kill imatinib-responsive and -unresponsive CML cell lines pretreated with zoledronate. Cytotoxicity was largely dependent on the granule exocytosis and partly on TRAIL pathways, was TCR-mediated, and was dependent on isoprenoid production by CML cells. Importantly, Vγ9Vδ2 T cells from patients with CML can be induced by zoledronate to develop antitumor activity against autologous and allogeneic zoledronate-treated leukemia cells, both in vitro and when transferred into immunodeficient mice in vivo.

We conclude that intentional activation of Vγ9Vδ2 T cells by zoledronate may substantially increase antileukemia activities and represent a novel strategy for CML adjunct immunotherapy.

Materials and Methods

Patients with CML and CML cell lines

Leukemia cell samples were obtained from 13 newly diagnosed patients with CML, followed at the Division of Haematology of the Ferrarotto Hospital in Catania, Sicily. Samples were collected at the time of diagnosis before patients started imatinib therapy. The study was approved by the ethical committees of the University Hospital of Palermo and Ferrarotto Hospital of Catania. Informed consent was obtained from all patients according to the Declaration of Helsinki. To obtain CML cells, whole blood samples were treated once or twice with RBC lysing buffer (Sigma-Aldrich, St. Louis, MO) for 2 min at room temperature, and then centrifuged at 350 × *g* for 7 min to recover white cells and discard lysed red cells. The pellets were resuspended in RPMI 1640 medium (Euroclone, Milan, Italy) supplemented with 10% FCS (Invitrogen, San Diego, CA), 2 mM L-glutamine, 20 nM HEPES, and 100 U/ml penicillin/streptomycin.

Imatinib-sensitive (K562S) or -resistant (K562R, KCL22R, and LAMA84R) CML cell lines were a gift of Prof. Carlo Gambacorti-Passerini (Clinical Research Unit, University of Milano Bicocca, Milan, Italy). The MM-1 cell line, expressing a BCR-ABL oncoprotein with two mutations in the TK domain (E255K and T315I), was obtained from a patient with CML in lymphoid blast crisis. All CML cell lines were grown in RPMI 1640 medium supplemented with 10% FCS and 2 mM L-glutamine.

For cytotoxic experiments, primary leukemic cells or cell lines were pretreated with zoledronate (0.5 μM final concentration) or imatinib (0.25 μM final concentration), both provided by Novartis Pharma (Basel, Switzerland). Pretreatment of CML cells (either lines or freshly isolated from patients) with imatinib or zoledronate, at the concentrations used in this study, does not have cytotoxic effect (Fig. 6*B*).

Abs and reagents

The following unconjugated or FITC-, PE-, PE-Cy5-, or APC-conjugated mAbs were obtained from BD Bioscience (San Diego, CA): anti-TCR Vδ2, anti-NKG2D, anti-Fas (CD95), anti-Fas Ligand (FasL; CD95L), anti–TNF-α, anti-MICA/B, anti-ULBP1, anti-ULBP2, anti-ULBP3, anti-CD16, anti-CD56, anti-CD161, anti-CD96, anti–DNAM-1 (CD226), and anti–nectin-2 (CD122). Additionally, the following purified mAbs were also used: anti-CD3 (blocking, MEM-57; a gift of Prof. Vaclav Horejsi, Institute of Molecular Genetics, Prague, Czech Republic), purified anti-TCR pan γδ (IMMU510; a gift of Dr. Marc Bonneville, Institut de Biologie, Nantes, France), anti-NKG2D (1D11 from eBioscience, San Diego, CA; 149810 from R&D Systems, Milan, Italy), anti–TNF-α (infliximab; a gift of Prof. Giovanni Triolo, Dipartimento Biomedico di Medicina Interna e Specialistica, Università di Palermo, Palermo, Italy), anti-CD95L (2C101; Vinci Biochem, Firenze, Italy), anti-TRAIL receptors TRAIL-R1 (DR4), TRAIL-R2 (DR5), TRAIL-R3 (LIT, DcR1) and TRAIL-R4 (TRUNDD, DcR2) (all kindly provided by Dr. Henning Walczak, Tumor Immunology Unit, Division of Medicine, Imperial College, London, U.K.). Concanamycin A (CMA) was purchased from Sigma-Aldrich, and atorvastatin was obtained from Calbiochem (Darmstadt, Germany).

Ex vivo expansion of Vγ9Vδ2 T cells

To expand human Vγ9Vδ2 T cells, PBMCs isolated from healthy adult donors or patients with CML by density gradient centrifugation using Ficoll-Hypaque (Pharmacia Biotech, Uppsala, Sweden) were cultured in RPMI 1640 supplemented with 10% FCS and antibiotics in the presence of 1 nM BrHPP (Phosphostim; a gift of Dr. Helene Sicard, Innate Pharma, Marseille, France) or 0.5 μM zoledronate (Novartis Pharma), added at day 0. After 48 h, recombinant human IL-2 (Novartis Pharma; 50 IU/ml final concentration) was added to the cultures, and the cells were supplemented with IL-2 every 3 d (18). Following 12–15 d culture, >90% of the cells expressed the Vγ9Vδ2 TCR, as determined by flow cytometry (FACS) analysis. The cells were harvested, and purified populations of Vγ9Vδ2 T cells were obtained by positive selection using magnetic microbeads conjugated to antihuman Vδ2 mAb (mouse IgG1κ, clone B6; Beckman-Coulter-Immunotech, Marseille, France) and immunomagnetic sorting (Miltenyi Biotec, Bergisch Gladbach, Germany). Isolated cells consisted of >98% Vγ9Vδ2 T cells, as determined by FACS analysis, and cell viability exceeded 95% as determined by trypan blue exclusion.

Proliferation assay

PBMC were labeled with CFSE (Molecular Probes, Eugene, OR) and cultured with 1 nM BrHPP or 0.5 μM zoledronate and 20 U/ml IL-2. After 7 d, the number of Vγ9Vδ2$^+$ T cells among CD3$^+$ cells was determined by FACS (19). The absolute number of Vγ9Vδ2 T cells present in each culture was calculated according to the following formula: % Vγ9Vδ2 T cells × total number of viable cells/100. The Vγ9Vδ2 T cell expansion factor was then calculated by dividing the absolute number of Vγ9Vδ2 T cells in stimulated cultures by the number of Vγ9Vδ2 T cells cultured with IL-2 alone (19).

Cytotoxic assay and blocking studies

Purified Vγ9Vδ2 T cells were resuspended at the final concentrations of 1.5×10^6, 3×10^6, and 6×10^6 cells/ml, and 100 μl was then added to round-bottom polystyrene tubes together with CML cells (100 μl), to obtain the E:T ratios of 5:1, 10:1, and 20:1. Cytotoxicity was measured by FACS analysis using CFSE and propidium iodide (PI; both from Molecular Probes) as described in Ref. 20. Briefly, a total of 50 μl of CFSE were added to 1 ml target cell suspension (5×10^5 cells/ml) in PBS to obtain the final concentration of 2.5 μM CFSE. The cells were incubated for 10 min at 37°C and gently mixed every 5 min. At the end of incubation, 1 ml FBS was added to the cell suspension to stop the staining reaction, and the cells were centrifuged at 600 × *g* for 5 min at room temperature, washed twice with cold PBS, and resuspended in serum-free medium. Control tubes containing only labeled target cells and effector cells were also prepared to establish background levels of cells death. Tubes were gently mixed, centrifuged at 300 × *g* for 2 min, and incubated at 37°C in 5% CO_2 for 4 h. At the end of the incubation period, the tubes were placed on ice, and 20 μl PI (1 μg/ml) were added to each tube for 10–15 min in ice. Finally, 100 μl complete medium were added before acquisition on an FACSCalibur cytometer (BD Biosciences). The calculation of cytolytic activity was based on the degree of reduction of viable target cells (VTC) with the ability to retain CFSE and exclude PI (CFSE^{high} PI$^-$). In some experiments, blocking mAbs (all at the final concentration of 10 μg/ml) were used to evaluate the mechanisms of Vγ9Vδ2 T cell-mediated recognition and cytotoxicity of CML cell lines. To evaluate the contribution of zoledronate-induced accumulation of mevalonate metabolites to kill target cells, tumor cells were treated for 2 h with 5 μM atorvastatin, a selective upstream inhibitor of the mevalonate pathway. Target cells were then incubated with zoledronate 0.5 μM for 20 h, washed, and added to Vγ9Vδ2 T cells. To inhibit perforin-mediated cytotoxicity, Vγ9Vδ2 T cells were incubated with 15 nM CMA for 30 min at 37°C prior to coculture, without further washing. Pretreatment of Vγ9Vδ2 T cells or CML cells with CMA or atorvastatin, at the concentrations used in this study, did not have any cytotoxic effect.

Flow cytometry and flow cytometry-based measure of synaptic transfer

Expression of surface markers by CML target cells was determined by flow cytometry (FACS) analysis. Cells were incubated in U-bottom 96-well plates, washed twice in PBS containing 1% FCS, stained for 30 min at 4°C with labeled Abs according to manufacturers' recommendations, washed, and analyzed by flow cytometry on an FACSCalibur with the use of CellQuest software (BD Biosciences). Viable cells were gated by forward and side scatter, and the analysis was performed on 100,000 acquired events for each sample.

Trogocytosis was measured according to Ref. 21. Briefly, CML target cells were stained with PKH67 lipophilic fluorochrome (Sigma-Aldrich), and effector Vγ9Vδ2 T cells were stained with Orange-5-and-6-(4-chloromethylbenzoyl-amino-tetramethylrhodamine) (CMTMR; Molecular Probes). Cells were then mixed together and cocultured in 96-well U-bottom tissue culture plates at an E:T ratio of 2:1 in medium, at the final concentration of 6×10^6 cells/ml. Culture plates were then centrifuged at 300 × *g* for 1 min and incubated for 3 or 60 min at 37°C. Postincubations, cells were collected, washed twice with PBS/EDTA, and analyzed on an FACS Calibur flow cytometer to detect trogocytosis. Data are expressed as PKH67 mean fluorescence intensity (MFI) of Orange-CMTMR–positive Vγ9Vδ2 T cells (21).

Confocal microscopy analysis of synaptic transfer and viability

Confocal microscopy analysis was carried out as described in Ref. 22. PKH67-stained CML cell lines and Orange-CMTMR–stained Vγ9Vδ2 T cells were coincubated for 1 h at 37˚C, as described above, at an E:T ratio of 5:1. The cells were then gently resuspended and plated on Lab-Tek chambered coverglass (VWR) previously coated with poly-D-lysine hydrobromide (Sigma-Aldrich) and incubated at 37˚C in a 5% CO_2 atmosphere. Postadhesion, cells were washed with PBS, fixed with PBS/2% p-formaldehyde, and slides mounted with PBS/2% diazobicyclooctane solution. Samples were examined using a Carl Zeiss LSM 410 confocal microscopy and AIM Imaging Software (Carl Zeiss, Oberkochen, Germany).

In vivo bioluminescent imaging of leukemia progression in SCID mice

The in vivo antitumor activity of Vγ9Vδ2 T cells was assessed according to previously published methods (23, 24). Briefly, 10^6 MM1 CML cell lines stably expressing firefly luciferase and GFP were injected i.v. at day 0, in groups of 8–14-wk-old nucleotide-binding oligomerization domain (Nod)/SCID mice (six mice per group; Charles River, Milan, Italy). A group of mice received Vγ9Vδ2 T cells (2×10^7/mouse) previously expanded and activated in vitro from PBMCs of patients with CML every 14 d, starting from day 1, together with 2 μg zoledronate i.p. (Novartis Pharma). These mice also received i.p. 30,000 IU IL-2 weekly. Control groups consisted of mice receiving IL-2 and zoledronate but not Vγ9Vδ2 T cells, or Vγ9Vδ2 T cells and IL-2 but not zoledronate, and Vγ9Vδ2 T cells and zoledronate but not IL-2. At day 42, the experiments were terminated and mice were sacrificed. All mice were analyzed on a weekly basis by in vivo imaging (Biospace Lab, Cambridge, MA) upon i.p. injection (100 μl) of D-luciferin (40 mg/ml) (Sigma-Aldrich). Photon signals were quantified using the M3 vision software for image analysis (Biospace Lab). The quantification of signal intensity was calculated as the sum of all detected photon flux counts within a uniform region of interest, manually selected during data postprocessing. The autobioluminescence, generated by residual food within the teeth and feces, was excluded and indicated by a yellow circle.

Mouse body weight was measured weekly, and animals suffering from wasting (loss of over 20% of initial body weight) were sacrificed.

Statistics

The two-tailed Student *t* test was used to compare significance of differences between groups. Data from experiments in Fig. 6 were compared using one-way ANOVA with Kruskal-Wallis multiple comparison test using Instat software (version 3.05; GraphPad, San Diego, CA). The p values <0.05 were considered statistically significant.

Results

Capture of CML cells membrane by Vγ9Vδ2 T lymphocytes

Trogocytosis is a straightforward assay to measure Vγ9Vδ2 T lymphocytes ability to conjugate with target cells into lytic synapses (22). Vγ9Vδ2 T lymphocytes cell lines trogocytose B and T cell lymphoma and leukemia cell lines, as well as the imatinib-sensitive K562S CML cell line (25). When coincubated with either imatinib-sensitive K562S or imatinib-resistant K562R, LAMA84R, and KCL22R CML cells labeled with the membrane fluorochrome PKH67, purified and ex vivo expanded Vγ9Vδ2 T cells also captured fragments of their membranes. Composite data with all tested CML cell lines are shown in Fig. 1*A*, and a representative experiment out of 10 with the LAMA84R cell line is shown in Fig. 1*B*. Pretreatment of CML cell lines with zoledronate significantly enhanced trogocytosis by Vγ9Vδ2 T lymphocytes over a 60-min coincubation period, as demonstrated by the shift of PKH67 intensity (MFI) in Vγ9Vδ2 T lymphocytes. In the same experiments, the MFI of Vγ9Vδ2 T cells did not shift significantly when CML cell lines were pretreated with imatinib, indicating that the nibbling of imatinib-treated CML cells is only very marginal if compared with that of zoledronate-treated CML cell lines. Similar results were obtained with ex vivo-expanded Vγ9Vδ2 T cells derived from three healthy donors (HDs) and four patients with CML (data not shown).

Confocal microscopy illustrated the capture of CML cells membrane fragments by Vγ9Vδ2 T lymphocytes that were in contact with the latter. Vγ9Vδ2 T lymphocytes were loaded with Orange-CMTMR and coincubated for 60 min with CML cell lines previously labeled with PKH67. Fig. 1*C* (representative results with the LAMA84R line, out of seven different experiments carried out with all four tested CML lines) shows that PKH67 was localized on the membrane of the conjugated Vγ9Vδ2 T lymphocytes, whereas the PKH67 membrane fluorochrome was absent prior to cell contact (not shown).

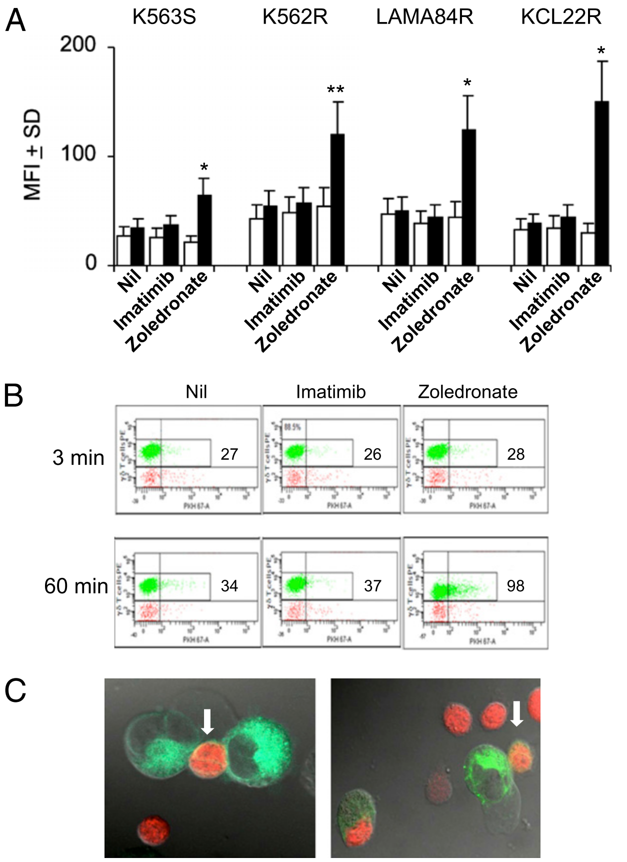

FIGURE 1. Capture of CML cell membrane fragments by Vγ9Vδ2 T cells. Four different CML cell line cells were treated with zoledronate or imatinib or with medium as a control for 24 h. Cells were labeled with the PKH67 fluorochrome and incubated for 3 or 60 min with Vγ9Vδ2 T cells and analyzed by flow cytometry. Shown are the PKH67 MFI ± SD of Vγ9Vδ2 T cells. *A*, Cumulative data at 3 (open columns) and 60 (filled columns) min from eight different experiments. *B*, Representative flow cytometry panels with the LAMA84R cell line. Numbers in each panel indicate the PKH67 MFI. *C*, Confocal images showing membrane capture of the PKH67-labeled LAMA84R (green) cell line pretreated with zoledronate and imatinib by CMTMR-stained Vγ9Vδ2 T cells (red) after 60 min of coincubation. *$p < 0.001$; **$p < 0.05$ when compared with control CML cells treated with medium.

Therefore, from this first set of experiments, we conclude that: 1) purified and ex vivo-expanded Vγ9Vδ2 T lymphocytes bind and trogocytose CML cell lines; and 2) trogocytosis is enhanced by previous treatment of CML cell lines with zoledronate.

Zoledronate sensitizes CML cell lines to Vγ9Vδ2 T cell cytotoxicity

Because trogocytosis mediated by Vγ9Vδ2 lymphocytes is associated with lytic synapses (25), we tested cytotoxic activity of ex vivo-expanded Vγ9Vδ2 T cells from HDs against different CML cell lines. All of the tested Vγ9Vδ2 T lymphocyte lines used in this study efficiently killed the standard target Daudi Burkitt lymphoma cell line, as well as the colon cancer DLD-1 cell line but not the normal colon CCL-241 cell line. Fig. 2*A* shows representative experiments with Vγ9Vδ2 T cell from two HDs (HD 1 and HD 3).

We assessed the ability of Vγ9Vδ2 T cells to kill imatinib-sensitive (K562S) and -resistant (K562R, KCL22R, and MM1) CML cell lines. Untreated CML cell lines were weakly sensitive to Vγ9Vδ2 T cell cytotoxicity, regardless of their responsiveness to imatinib. Lysis percentages ranged from 12–19%, at an E:T ratio of 20:1 without significant differences at other E:T ratios (Fig. 2*B*). The poor cytotoxic activity toward CML cell lines was not an intrinsic property of the γδ T cells, because the Daudi and DLD-1 tumor cell lines were recognized and killed efficiently by the same Vγ9Vδ2 T cell lines (Fig. 2*A*). Pretreatment with zoledronate for 24 h was sufficient to render both imatinib-sensitive and -resistant CML cell lines highly susceptible to Vγ9Vδ2 T cell killing, increasing levels of cytotoxicity from 12% to 46% for K562S, from 19% to 61% for K562R, and from 13% to 56% for KCL22R, at an E:T ratio of 20:1. Treatment of target cells with imatinib alone did not increase sensitivity of CML cell lines to Vγ9Vδ2 T cell-mediated cytotoxicity. Failure to measure lysis of the imatinib-sensitve K562S cell line might be due to the use of an imatinib concentration 5-fold lower than that shown to inhibit 50% growth of CML cell lines over a 48-h period (14–16). Fig. 2*B* shows representative experiments with expanded Vγ9Vδ2 T cells from one HD (HD 3). Notably, neither zoledronate nor imatinib caused drug-associated cytotoxicity on all tested CML cell lines (data not shown). Confocal microscopy analysis (Fig. 2*C*) illustrates apoptosis of the representative K562R CML cell line pretreated or not with zoledronate by Vγ9Vδ2 T lymphocytes that were in contact with the latter.

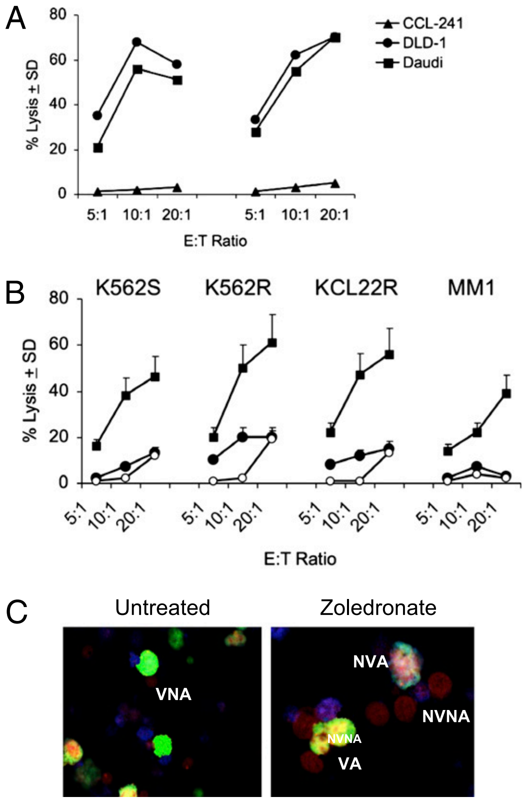

FIGURE 2. Vγ9Vδ2 T cell-mediated lysis of CML cell lines sensitive or resistant to imatinib. *A*, Specific lysis of Daudi Burkitt lymphoma cells (squares), colon cancer DLD-1 cells (circles), and normal colon CCL-241 cells (triangles) by Vγ9Vδ2 T cell line. Data shown are the mean ± SD. *B*, Cytotoxic activity of Vγ9Vδ2 T cells against untreated (open circles), imatinib-treated (filled circles), or zoledronate-treated (filled squares) CML target cells, both sensitive (K562S) and resistant (K562R, KCL22R, and MM1) to imatinib. Percentages of specific lysis at a given E:T ratio are shown. Data shown are the mean values of cytotoxicity ± SD from one representative experiment of seven independent experiments performed with different Vγ9Vδ2 T cell lines. *C*, Confocal image showing Vγ9Vδ2 T cells (blue) coincubated for 4 h with K562R CML cells pretreated or not with zoledronate and stained with acridine orange/ethidium bromide solution to detect apoptosis and changes in cells morphology; four types of tumor target cells were detected: 1) viable non apoptotic (NA), 2) viable apoptotic (VA), 3) nonviable apoptotic (NVA), and 4) nonviable nonapoptotic cell (NVNA).

We also tested the cytotoxic ability of Vγ9Vδ2 T cells against another peculiar CML cell line, called MM1, which expresses both the E255K and T315I mutations, thereby exhibiting resistance to all available TK inhibitors. Untreated MM1 cells were poorly sensitive to Vγ9Vδ2 T cell lysis (2% at a T:T ratio of 20:1) but pretreatment of these targets with zoledronate for 24 h consistently increased Vγ9Vδ2 T cell-mediated cytotoxicity, with values reaching almost 40% (Fig. 2*B*). As expected, pretreatment with imatinib alone did not sensitize these cells to lysis.

Thus, zoledronate sensitizes CML cell lines to Vγ9Vδ2 T cell-mediated cytotoxicity.

CML cell lines constitutively express molecules involved in killing by Vγ9Vδ2 T cells

To determine possible mechanisms involved in Vγ9Vδ2 T cell-mediated cytotoxicity, we examined CML cell surface expression of MICA/B molecules, ULBPs, nectin (CD122), Fas (CD95), DR4 (TRAIL-R1), and DR5 (TRAIL-R2) death receptors as well as DcR1 (TRAIL-R3) and DcR2 (TRAIL-R4) decoy receptors, that lack a functional death domain and cannot transduce a proapoptotic signal. As depicted in Fig. 3, both imatinib-sensitive and -resistant CML cell lines constitutively express all these molecules, with the exception of DR5 receptor, which was not expressed at detectable levels by any of the tested cell lines. Although expression of the above-indicated molecules varied among the cell lines, no variation was observed following exposure for 24 h to zoledronate. We only found a slight, nonsignificant reduction of the expression of DcR1 and DcR2 only on the imatinib-sensitive K562S cell line post-treatment with zoledronate (data not shown).

Vγ9Vδ2 T cells kill zoledronate-sensitized CML cell lines via TCR-mediated recognition and the perforin and TRAIL pathways

It has been proposed that human Vγ9Vδ2 T cells trigger several distinct pathways for killing tumor cells (26). These pathways include secretion of proinflammatory cytokines and proapoptotic molecules or on cell contact-dependent lysis through an NK-like or TCR-dependent signal. Mechanisms responsible for Vγ9Vδ2 T cell recognition and killing of zoledronate-sensitized CML cells were assessed by individually blocking TCR, NK receptor, perforin, Fas, and TRAIL pathways (Fig. 4). Cytotoxicity of both imatinib-sensitive and -resistant CML cell lines was inhibited at the greatest extent by anti-pan γδ TCR (55% inhibition in K562S, 77% in K562R, and 71% in KCL22R), indicating a TCR-mediated recognition and killing. NKG2D seemed instead to play a minor role in Vγ9Vδ2 T cell cytotoxicity against CML cells, with no variation (K562R and KCL22R) or a small inhibition (15% in K562S) observed posttreatment with an anti-NKG2D Ab. In addition, Vγ9Vδ2 T cell recognition of zoledronate-sensitized CML cell targets was assessed in the presence of atorvastatin, which inhibits 3-hydroxy-3-methyl-glutaryl-CoA reductase and prevents zoledronate-mediated accumulation of endogenous IPP (26, 27). Atorvastatin caused significant inhibition of the killing of zoledronate pretreated CML cell lines, indicating that production of mevalonate metabolites is not redundant for CML cell recognition and killing by Vγ9Vδ2 T cells. Fig. 4 shows cumulative data performed in seven different experiments.

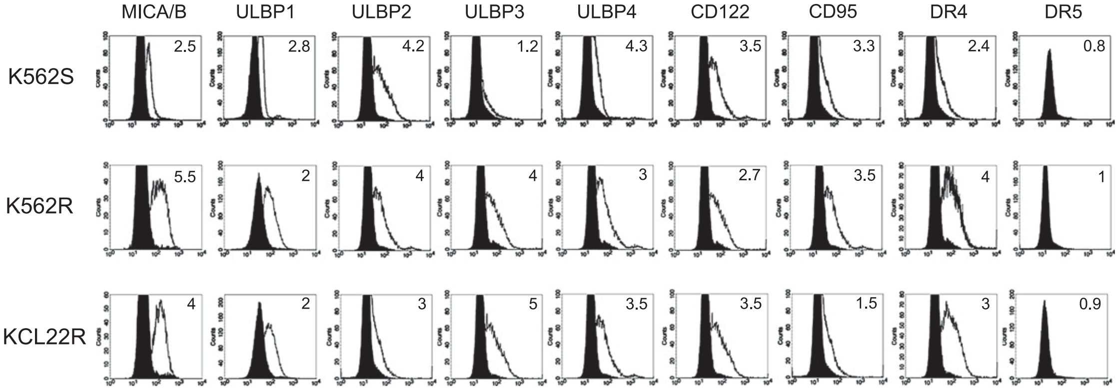

FIGURE 3. Phenotype of CML cell lines. Representative overlay histograms showing constitutive surface expression (open histograms) of MICA/B, ULBP1-4, CD122 (nectin), CD95 (Fas), DR4 (TRAIL-R1) and DR5 (TRAIL-R2) molecules on CML cell lines against appropriate control Ig isotypes (filled histograms). The numbers indicate the *x*-fold increase in median fluorescence intensity over the isotype control as determined on a 4-log scale.

To further elucidate the mechanisms responsible for killing of zoledronate-sensitized CML cell lines by Vγ9Vδ2 T cells, we individually inhibited the granule exocytosis-, TRAIL-, TNF-α, and FasL-mediated pathways. Killing-inhibition experiments using CMA revealed (Fig. 4) that Vγ9Vδ2 T cell cytotoxicity of zoledronate-pretreated CML targets was mainly mediated by the perforin pathway (means of 82–86% inhibition using CMA). Addition of Abs against TRAIL-R1 and -R2 caused a 37% and a 25% inhibition of the killing of zoledronate-treated K562S and K562R, respectively, but had no effect on the death of zoledronate-treated KCL22R cells, indicating that TRAIL played a variable role in the killing activity of Vγ9Vδ2 T cells toward CML cell lines. Finally, Abs to FasL and TNF-α failed to inhibit the cytotoxicity of all tested zoledronate-sensitized CML targets (Fig. 4).

Vγ9Vδ2 T cells from patients with CML kill primary leukemic cells freshly isolated from patients with CML

Preliminarily, we analyzed the size and functionality of Vγ9Vδ2 T cells in the peripheral blood of patients with CML by measuring their ability to proliferate and differentiate, following exposure to zoledronate and IL-2. Ex vivo stimulation with zoledronate and IL-2 induced significant increase of the frequency of $V\delta2^+$ T cells within PBMCs from patients with CML and also promoted efficient Vγ9Vδ2 T cell expansion (9–420-fold expansion) (Fig. 5*A*, 5*B*). Moreover, as shown in Fig. 5*C*, in cultures of PBMC from patients with CML stimulated by zoledronate and IL-2, most Vγ9Vδ2 T cells displayed an effector memory ($CD27^-CD45RA^-$) phenotype, like stimulated PBMC cultures from HDs.

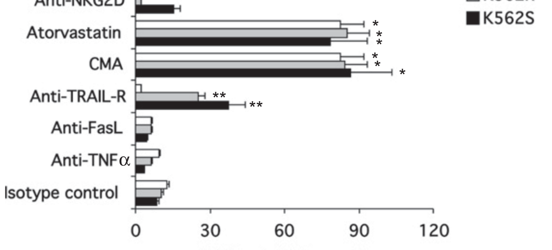

FIGURE 4. Mechanisms of Vγ9Vδ2 T cell killing of CML target cells. Vγ9Vδ2 T cell lines were cultured with zoledronate-treated CML cells at an E:T ratio of 20:1 in the presence of blocking Abs to the γδ TCR, NKG2D, FasL, TNF-α, TRAIL-R1 or -R2, atorvastatin, or CMA. Data are mean ± SD of seven different experiments carried out in triplicate. $*p < 0.001$; $**p < 0.02$ when compared with cytotoxicity carried out in the absence of inhibitors.

Importantly, Vγ9Vδ2 T cells from patients with CML can be induced by zoledronate to develop antitumor activity against CML lines and autologous and allogeneic, zoledronate-treated, leukemia cells taken from patients with CML at the time of diagnosis and in the absence of any treatment.

Results show that Vγ9Vδ2 T cells from patients with CML failed to kill both CML lines and their own tumor cells, but treatment of target cells with zoledronate significantly increased Vγ9Vδ2 T cell cytotoxicity in 10 out of the 13 tested patients with CML (6–63% of cytotoxicity), while slightly increasing lysis in the other three patients (2–5%, 1–6%, and 4–7%, respectively; data not shown). Fig. 6*A* shows representative results of the cytotoxic activity of Vγ9Vδ2 T cells from two lymphocyte-mediated cytotoxicity patients (CML 7 and CML 12) toward autologous and allogeneic leukemia cells, as well as toward CML cell lines. Notably, pretreatment of cells from patients with CML with zoledronate did not have cytotoxic effects per se on CML tumor cells, as demonstrated by double staining with CFSE and PI (Fig. 6*B*).

Therefore, zoledronate enhances the susceptibility of both primary and immortalized CML cells to Vγ9Vδ2 T cell-mediated cytotoxicity.

Vγ9Vδ2 T cells killing leukemia cells in vivo

To evaluate the potential of immunotherapy strategies, we used a previously published model of transplantation of human tumors into lymphopenic Nod/SCID mice (23, 24) and added bioluminescent analysis of tumor development, which allows early detection of tumors and temporal evaluation throughout the course of treatment in live animals and real-time. Four weeks postinjection of MM1 cells, mice that had received activated and expanded Vγ9Vδ2 T cells, zoledronate, and IL-2 showed significantly reduced tumor load compared with control mice (Fig. 7*A*). Furthermore, whereas most controls had to be sacrificed at day 28 due to excessive body weight loss, Vγ9Vδ2 T cell-treated animals resisted wasting for longer up to day 84 (data not shown). Fig. 7*B* show typical results obtained in two mice receiving expanded Vγ9Vδ2 T cells, zoledronate, and IL-2 and in two control mice who received IL-2 and zoledronate, but not Vγ9Vδ2 T cells. These results attest the capacity of Vγ9Vδ2 T cells to induce anti-tumor responses in vivo, and support their potential application in conjugation with zoledronate and IL-2 in clinical cancer settings.

Discussion

Although the use of imatinib has represented an important advance for the treatment of CML, 20–30% of patients treated with the drug fail to achieve a complete cytogenetic response, and even patients that exhibit an optimal response may subsequently present

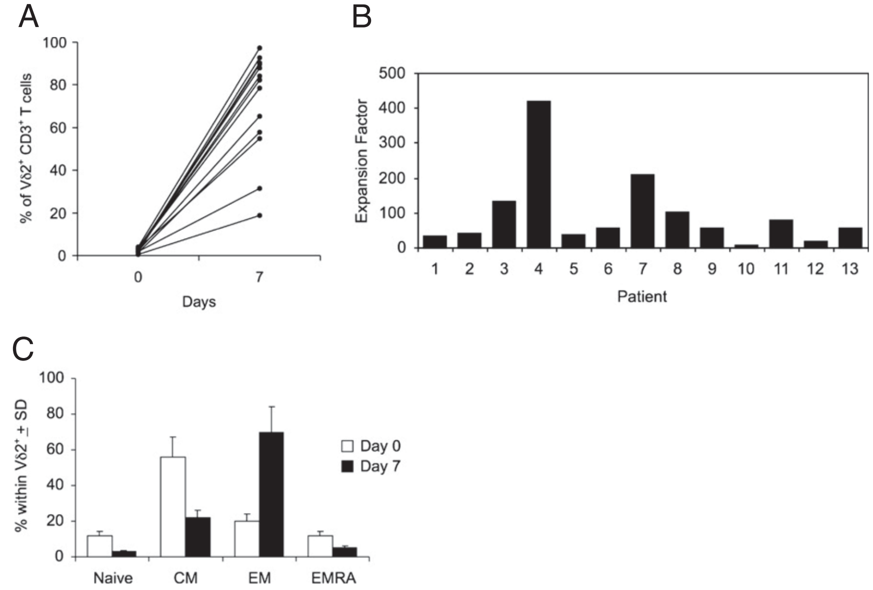

FIGURE 5. Functional analysis of Vγ9Vδ2 T cells from patients with CML. Kinetics of Vδ2$^+$ CD3$^+$ cells frequency within PBMC of 13 patients with CML (*A*) and corresponding fold amplifications (*B*) relative to day 0 were measured following specific activation with IL-2 and zoledronate or IL-2 alone as a control. *C*, Analysis of the memory status of Vγ9Vδ2 T cells generated in the same experiment. The percentage of naive (CD27$^+$CD45RA$^+$), central memory (CM; CD27$^+$CD45RA$^-$), effector memory (EM; CD27$^-$CD45RA$^-$), and terminally differentiated effector memory (EMRA; CD27$^-$CD45RA$^+$) cells are indicated within each subset. Data shown are the mean percent values ± SD of one representative of at least three experiments performed by using PBMC samples from 13 patients with CML.

a relapse of their disease. Moreover, ~15% of patients treated with imatinib will only obtain a suboptimal response [i.e., a temporary state that will likely require a higher dose of imatinib or a change in drug treatment (4)]. In this context, additional immunotherapy may provide an opportunity to either improve clinical responses in patients who are resistant to imatinib or convert suboptimal responders in optimal responders.

Zoledronate is a third-generation N-BP already used in the treatment of cancer-related bone complication (28–30). Moreover, zoledronate inhibits in vitro proliferation, induces apoptosis of imatinib-resistant leukemia cells, and augments the anti-Ph$^+$ leukemia activity of imatinib (15, 16, 31). However, zoledronate concentrations used in these in vitro studies are in the micromolar range (20–50 μM zoledronate was generally employed), although in humans treated with the drug, the maximal plasma concentrations range from 0.5–5 μM, depending on the dosage and duration of infusion (32, 33). Moreover, the high micromolar concentrations required in the in vitro experiments present a considerable risk for the toxicity to nonleukemic cells.

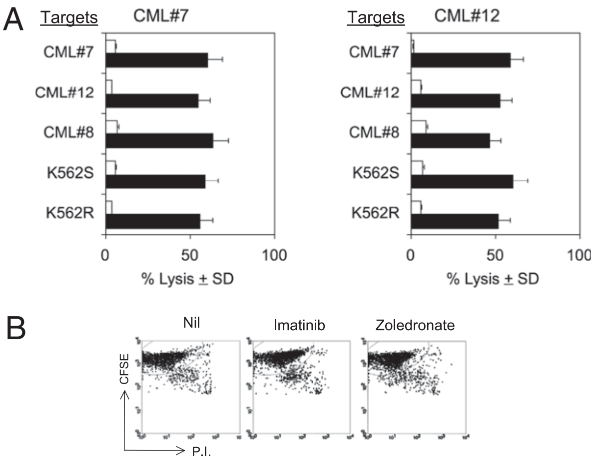

FIGURE 6. Vγ9Vδ2 T cells from patients with CML kill freshly isolated, zoledronate-treated, autologous and allogeneic leukemia cells and CML cell lines. Leukemia cells were purified from patients with CML at the time of diagnosis and used for cytotoxic experiments with Vγ9Vδ2 T cells from patients with CML. *A*, Shown is specific lysis obtained with ex vivo-expanded Vγ9Vδ2 T cells from two patients with CML (CML 7 and CML 12) against leukemia cells of three patients with CML (CML 7, CML 8, and CML 12) and CML cell lines (K562S and K562R), untreated (open bars) or pretreated with zoledronate (filled bars). Data show the mean values ± SD of a representative experiment out of three performed in triplicate. *B*, Pretreatments with imatinib or zoledronate alone did not induce any cytotoxic effect per se, as indicated by CFSE versus PI staining.

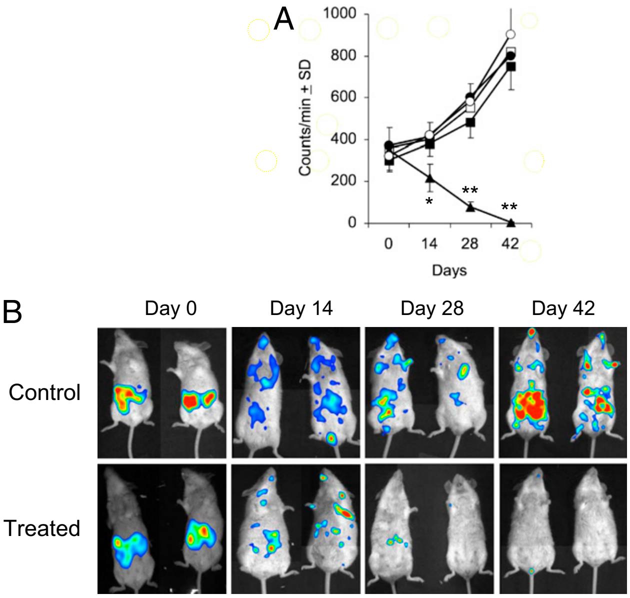

FIGURE 7. In vivo leukemia cell-killing activity of Vγ9Vδ2 T cells. Bioluminescent imaging of Nod/SCID mice injected with luciferase MM1 leukemic cell lines and treated with zoledronate and IL-2, as described in *Materials and Methods* (*B*). *A*, Image quantification of photon signals (tumor load) collected at the indicated time points. Data in this figure are representative of three independent experiments. Filled triangles indicate mice treated with zoledronate, IL-2, and with expanded and activated Vγ9Vδ2 T cells. Open circles indicate untreated control mice, receiving only leukemic cells. Open squares indicate mice treated with zoledronate and IL-2. Filled circles indicate mice treated with zoledronate and with expanded and activated Vγ9Vδ2 T cells. Filled squares indicate mice treated with IL-2 with expanded and activated Vγ9Vδ2 T cells. $*p < 0.02$; $**p < 0.001$ when compared with control mice.

In this study, we used purified and ex vivo-expanded Vγ9Vδ2 T cell lines to kill CML cells, and we found that pretreatment of target cells with zoledronate alone or in combination with imatinib significantly increased their trogocytosis and killing by Vγ9Vδ2 T lymphocytes. Similar to previous reported data (25), we found that trogocytosis perfectly matches cytotoxicity measured on target cells, further indicating that target cell death relies much more upon contact with Vγ9Vδ2 T lymphocytes and subsequent involvement of perforin and, at a lesser extent, TRAIL. Both imatinib-sensitive and -resistant CML cells pretreated with zoledronate for 24 h were efficiently killed by Vγ9Vδ2 cells. Interestingly, also the leukemia cell line MM1, which carries a double mutation in the TK domain that confers resistance to any available TK inhibitor, was efficiently killed by Vγ9Vδ2 T cells when pretreated with zoledronate.

Previous studies have demonstrated that zoledronate synergizes with imatinib to inhibit Ph^+ primary leukemic cell growth (15, 16), whereas zoledronate or imatinib, either alone or in combination, were not effective against leukemic cells harboring the E255K or T315I mutations. Conversely, in our experiments, pretreatment of MM1 cells with zoledronate were allowed to obtain significant cytotoxic values reaching ~40%.

Human Vγ9Vδ2 T cells recognize phosphoantigens, which are metabolites of isoprenoid biosynthetic pathways (34, 35), and the more recently described ATP synthase-F1/apolipoprotein A-1 complex that, unlike in normal cells, is ectopically expressed on the surface of hemopoietic and solid cancer cells (36). Moreover, Vγ9Vδ2 T cell activity is tightly regulated by NK-like receptors, and previous studies have indicated the importance of NKG2D-MICA/B interactions for tumor cell recognition and cytotoxicity by Vγ9Vδ2 T cells (9, 37, 38). It has been suggested that treatment of tumor cells with zoledronate leads to the intracellular accumulation of phosphoantigens (typically IPP), favoring recognition and killing of tumor cells by the reactive Vγ9Vδ2 T lymphocytes (14, 39–41). In our study, Vγ9Vδ2 T cell recognition and killing of zoledronate-treated CML target cells was TCR-mediated and depending on the synthesis of isoprenoid intermediates, because preventing accumulation of IPP and/or other endogenous phosphoantigens posttreatment with atorvastatin significantly impaired Vγ9Vδ2 T cell cytotoxicity, thus indicating that the sensitizing effect of zoledronate correlates with increased expression/production of mevalonate metabolites. A recent paper by Li and coworkers (40) used siRNA to provide support of the concept that increased intracellular IPP levels are instrumental in Vγ9Vδ2 T cell activation by tumor lines, which so far has been based on a correlation between IPP levels and Vγ9Vδ2 T cell activation as well as by observations with enzyme inhibitors, such as 3-hydroxy-3-methyl-glutaryl-CoA-reductase inhibitors (e.g., mevastatin) and farnesyl pyrophosphate synthase inhibitors (e.g., aminobisphosphonates). Although ATP synthase expression can also be detected on CML cell lines used in this study (data not shown), because currently available anti-ATP synthase Abs are not reliable for inhibition experiments, we could not evaluate the contribution of ATP synthase to Vγ9Vδ2 T cell-mediated recognition and killing of CML target cells. Moreover, NKG2D and other NK ligands/receptors do not appear to contribute to the cytotoxicity of zoledronate-treated CML cells, because addition of specific blocking Abs failed to inhibit lysis, and treatment with zoledronate did not alter MICA/B, ULBPs, and nectin expression on the membrane of CML cells.

Previous Ab-blocking studies by Wrobel and coworkers (42) have shown three different patterns of tumor cells recognition and killing by Vγ9Vδ2 T cells: preferential involvement of the TCR, preferential involvement of NKG2D, or additive involvement of both. Moreover, and similar to the results reported in this study, the extent of inhibition of the cytolytic activity of Vγ9Vδ2 T cells by anti-NKG2D Abs did not correlate directly with the level of NKG2D ligand expression on the tumor cells or with the origin and progression stage of a tumor cell (42).

How far other molecules, such as the ectopically expressed F1-ATPase, which has been claimed to serve as the Vγ9Vδ2 TCR ligand expressed by Daudi tumor cells, are involved in IPP recognition remains unclear.

It is known that Vγ9Vδ2 T cells kill tumor targets via a number of mechanisms including death receptor/ligand interactions with TRAIL and FasL and release of perforin/granzymes. In theory, one or more of these pathways may be involved in the killing of CML cell lines. Although all CML cell lines evaluated in this study constitutively expressed TRAIL receptors and Fas, this expression did not initially translate into sensitivity to Vγ9Vδ2 T cell killing, as documented by the failure of specific blocking Abs to consistently inhibit cytotoxicity. Additionally, exposure of CML cell lines to zoledronate did not cause any variation of TRAIL receptors and Fas expression. However, TRAIL-mediated cytotoxicity may have instead a minor role because lysis of K562S was reduced ~35% after blocking interaction of TRAIL with its receptors, whereas lysis of the imatinib-resistant cell lines was only poorly (K562R) or not at all affected (KCL22R).

Irrespective of imatinib sensitivity or resistance of CML lines, CMA strongly inhibited cytotoxicity, indicating that zoledronate-treated targets are almost exclusively killed by perforin release by Vγ9Vδ2 T cells, consistent with previous findings of perforin/granzyme-dominated killing (43–46).

As it would be of interest to test in vivo the clinical efficacy of a Vγ9Vδ2 T cell-mediated immune therapy, we assessed the cytotoxic ability of Vγ9Vδ2 T cells on a limited number of cells taken from patients with CML at the time of diagnosis and before therapy. The results obtained showed that Vγ9Vδ2 T cells kill cells freshly isolated from patients with CML, but exclusively when cells were pretreated with zoledronate alone or in combination with imatinib.

Our findings, together with the attainment that zoledronate has antileukemic properties, firstly indicate that zoledronate-activated Vγ9Vδ2 T cells possess a promising cytotoxic activity against CML cells resistant to imatinib. On this basis, we would like to suggest the clinical utility of intentional in vivo activation of Vγ9Vδ2 T cells by zoledronate and low doses of IL-2 in those patients refractory to imatinib treatment alone, as recently performed in other hematological malignancies (10) and in prostate cancer (12). Even if potent cytotoxic Vγ9Vδ2 T cells may be generated from blood cells of patients with myeloma and lymphoma (8), sometimes proliferative responses of Vγ9Vδ2 T cells from patients with cancer turned out to be suppressed (47), thus accounting for tumor-induced anergy after chronic Vγ9Vδ2 T cell stimulation. This problem could be circumvented by administration of in vitro-expanded allogeneic Vγ9Vδ2 T cells from HDs, because they are not alloreactive and have not been involved in graft-versus-host reactions. Although clinical investigations are necessary to test in vivo the efficacy of in vitro skilled immune cytotoxicity protocol, both the Vγ9Vδ2 T cell transfer and the infusion of bisphosphonate drugs have been proven to be well tolerated (10, 12, 48–51). In this regard, data presented in this study support the proposal that zoledronate may prove a novel, safe, feasible, and efficacious means to activate in vivo γδ T cells and to sensitize CML cells to their cytotoxic activity; in turn, this could allow us to extend the life span of patients with CML and thereby to increase the window of the patients' availability for other more specific molecular approaches.

Acknowledgments

We thank Marc Bonneville, Vaclav Horejsi, Helene Sicard, Giovanni Triolo, Henning Walczak, and Carlo Gambacorti-Passerini for the generous gift of reagents and for reading the manuscript.

Disclosures

F.D. is a founding member of a University of Palermo spinout company (TetraPharm S.r.l.), in which he has a share of equity and for which he acts as scientific advisor in a nonexecutive capacity. F.D. is named inventor for several patents filed by TetraPharm S.r.l. on products that are related to those studied in this work.

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