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Efficient Killing of Human Colon Cancer Stem Cells by $\gamma\delta$ T Lymphocytes¹

Matilde Todaro,* Matilde D'Asaro,[†] Nadia Caccamo,[†] Flora Iovino,* Maria Giovanna Francipane,* Serena Meraviglia,[†] Valentina Orlando,[†] Carmela La Mendola,[†] Gaspare Gulotta,[§] Alfredo Salerno,[†] Francesco Dieli,^{2,3†} and Giorgio Stassi^{2*}

Colon cancer comprises a small population of cancer stem cells (CSC) that is responsible for tumor maintenance and resistant to cancer therapies, possibly allowing for tumor recapitulation once treatment stops. We previously demonstrated that such chemoresistance is mediated by autocrine production of IL-4 through the up-regulation of antiapoptotic proteins. Several innate and adaptive immune effector cells allow for the recognition and destruction of cancer precursors before they constitute the tumor mass. However, cellular immune-based therapies have not been experimented yet in the population of CSCs. Here, we show that the bisphosphonate zoledronate sensitizes colon CSCs to V γ 9V δ 2 T cell cytotoxicity. Proliferation and production of cytokines (TNF- α and IFN- γ) and cytotoxic and apoptotic molecules (TRAIL and granzymes) were also induced after exposure of V γ 9V δ 2 T cells to sensitized targets. V γ 9V δ 2 T cell cytotoxicity was mediated by the granule exocytosis pathway and was highly dependent on isoprenoid production by of tumor cells. Moreover, CSCs recognition and killing was mainly TCR mediated, whereas NKG2D played a role only when tumor targets expressed several NKG2D ligands. We conclude that intentional activation of V γ 9V δ 2 T cells by zoledronate may substantially increase antitumor activities and represent a novel strategy for colon cancer immunotherapy. *The Journal of Immunology*, 2009, 182: 7287–7296.

Colorectal cancer is the second leading cause of cancer-related death in the Western World. Nowadays, only a few cases of this cancer are found at an early stage, and available treatments, including surgery, chemotherapy, and/or radiation, are often not successful in completely eradicating the tumor.

For many years, colon tumor has been defined as the result of the proliferation of a single somatic cell in which multiple mutations had occurred. Particularly, alterations in adenomatous polyposis coli, β -catenin, *K-ras*, *DCC*, and *p53* genes have been involved in the multistep process of colon carcinogenesis (1) in which the colonic mucosa as it undergoes hyperproliferation and adenomatous change, becomes dysplastic, and sometimes undergoes malignant transformation (2).

It is now emerging that colon tumor could be generated by a population of cells displaying stemness features, the so-called cancer-initiating cells or cancer stem cells (CSC).⁴ These cells, which

comprise only a small fraction of total cells, express the cell surface marker CD133 (3–5), a five-transmembrane glycoprotein previously identified in hemopoietic (6) and neural stem cells (7). The CD133⁺ colon cancer cells have been shown to undergo long-term expansion without loss of their ability to reproduce the human original tumor phenotype thus, providing functional evidence for self-renewal and tumor-initiating capacity (5). Moreover, it has been demonstrated that within the tumor, the CD133 subpopulation is more resistant than differentiated primary cells to the conventional chemotherapeutic drugs and to putative innovative therapies such as those based on the use of TRAIL (5). This refractoriness, which would elucidate the recurrence of cancer and eventually the metastasis formation even when the chemotherapy has caused the primary tumor mass to disappear, is due to the release of IL-4 by CD133⁺ colon cancer cells (5). Hence, blockage of the IL-4-activated signaling pathway overcomes this resistance through the reduction of prosurvival molecules (5). Clinical studies have recently shown that adding immune therapy to chemotherapy has survival benefits in comparison with chemotherapy alone (8), an example being in the setting of mAb-chemotherapy combination therapy (9–11). Moreover, chemotherapeutic agents can sensitize tumors to immune cell-mediated killing (reviewed in Ref. 12), for instance increasing sensitivity of tumor cells to subsequent cytotoxicity by T cells via up-regulation of death receptors DR5 and Fas, ligands to TRAIL and CD95L (FasL), respectively (13). Most current immunotherapeutic approaches aim at inducing antitumor response stimulating the adaptive immune system, which is dependent on MHC-restricted $\alpha\beta$ T cells. However, loss of MHC molecules is often observed in

*Department of Surgical and Oncological Sciences, University of Palermo, Palermo, Italy; [†]Department of Biopathology and Biomedical Methodologies, University of Palermo, Palermo, Italy; and [§]Department of Emergency General Surgery and Organ Transplants, University of Palermo, Palermo, Italy

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² F.D. and G.S. contributed equally to this work.

³ Address correspondence and reprint requests to Dr. Francesco Dieli, Dipartimento di Biopatologia e Metodologie Biomediche, Università di Palermo, Corso Tukory 211, 90134 Palermo, Italy. E-mail address: dieli@unipa.it

⁴ Abbreviations used in this paper: CSC, cancer stem cell; FasL, Fas ligand; N-BP, aminobisphosphonate; BLT, *N*^α-carbobenzoyl-L-lysine-thiobenzyl; IPP, isopentenyl

pyrophosphate; EF, expansion factor; CMA, concanamycin A; ESA, epithelial-specific Ag; MIC, major histocompatibility complex class I-related molecules; PI, propidium iodide.

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cancer cells, rendering tumor cells resistant to $\alpha\beta$ T cell-mediated cytotoxicity (14, 15). $\gamma\delta$ T cells exhibit potent MHC-unrestricted lytic activity against different tumor cells in vitro, suggesting their potential utility as anticancer therapy. Moreover, $\gamma\delta$ T cells have been consistently identified and isolated from tumor infiltrating lymphocytes in various types of cancer, including prostate carcinoma (16, 17).

Among $\gamma\delta$ T cells, human V γ 9V δ 2 T cells can be activated by a variety of nonpeptide phosphoantigens or by agents which cause their accumulation within cells: among these latter are aminobisphosphonates (N-BP), (18). N-BPs, in addition to their effects of inhibiting osteoclastic bone resorption (19), exhibit direct antitumor activity by both inhibiting proliferation and inducing apoptosis in tumor cells (20). Their unique ability to render tumor cells susceptible to V γ 9V δ 2 T cell attack makes these drugs particularly interesting candidates for use in T cell therapy (21–25).

Once recognized, N-BPs lead to $\gamma\delta$ T cells activation, proliferation and acquisition of effector functions. Accordingly, the N-BP zoledronate induces V γ 9V δ 2 T cell distribution in cancer patients in vivo, decreasing the naive and memory subsets and expanding the IFN- γ -producing effector phenotype (26). More recently, we conducted a phase I clinical trial to determine the antitumor effects of the single or combined administration of zoledronate plus IL-2 in patients with metastatic hormone-refractory prostate cancer, concluding that the combined treatment is able to shift $\gamma\delta$ T cells toward an activated effector memory-like state, producing IFN- γ , TRAIL, and perforin, and consequently improving the prognosis of treated patients (27).

In this study, we have assessed whether zoledronate can be used to sensitize malignant colon CSC targets to V γ 9V δ 2 T cell cytotoxicity. We demonstrate the ability of V γ 9V δ 2 T cells to efficiently kill zoledronate-sensitized colon CSCs. We also show that mechanisms, through which cytotoxicity occurs, involve the V γ 9V δ 2 TCR, the granule exocytosis pathway and, partially, modification of the mevalonate pathway.

Materials and Methods

Colon specimens

Colon cancer tissues were obtained in accordance with the ethical standards of the institutional committee of human experimentation from patients undergoing a colon resection for colon adenocarcinoma. Histological diagnosis was based on microscopic features of carcinoma cells determining the histological type and grade.

Colon CSC purification and culture

Cancer tissues were extensively washed in saline buffer containing antibiotics and incubated overnight in DMEM/F12 (Life Technologies) containing penicillin (500 IU/ml), streptomycin (500 μ g/ml), and amphotericin B (1.25 μ g/ml), from Life Technologies. Enzymatic digestion was performed using collagenase (1.5 mg/ml; Life Technologies) and hyaluronidase (20 μ g/ml; Sigma-Aldrich) in DMEM containing antibiotics/antimycotics for 1 h. Recovered cells were then cultured in serum-free medium (DMEM/F12) supplemented with 6 mg/ml glucose, 1 mg/ml NaHCO₃, 5 mM HEPES, 2 mM L-glutamine, 4 μ g/ml heparin, 4 mg/ml BSA, 10 ng/ml basic fibroblast factor, 20 ng/ml EGF, 100 μ g/ml apotrasferrin, 25 μ g/ml insulin, 9.6 μ g/ml putrescin, 30 nM sodium selenite anhydrous, and 20 nM progesterone (Sigma-Aldrich) to a final concentration of 3×10^5 cells/ml. These culture conditions select for immature tumor cells that slowly proliferate, giving rise, within 2–3 mo, to tumor cell aggregates, called spheres. Sphere-forming cells can be propagated by enzymatic dissociation (3 mM EDTA, 50 nM DTT in PBS) of spheres, followed by a replating of single cells and residual small-cell aggregates in fresh serum-free medium (5). These cells have been treated with 200 nM zoledronate (Novartis) for 24 h at 37°C.

To obtain differentiated colon cancer cells to be compared with immature cells, sphere cells were cultured for 1–2 wk in differentiating media (DMEM supplemented with 10% FBS). Tumorigenicity was evaluated by

s.c. implantation of either disaggregated colon cancer sphere cells or sphere-derived differentiated cells (5). Differentiated colon cancer DLD-1, SW620, and SW403 cells lines and the normal colon CCL-241 cell line (American Type Culture Collection) were maintained in DMEM containing antibiotics and 10% heat-inactivated FCS (Life Technologies). All cell cultures were conducted at 37°C in a 5% CO₂ humidified incubator.

V γ 9V δ 2 T cell culture

To expand human V γ 9V δ 2 T cells, PBMC were cultured in RPMI 1640 supplemented with 10% FCS and antibiotics, in the presence of 50 nM zoledronate plus recombinant human IL-2 (20 IU/ml; Novartis) added at day 0 and every 3 days (28). After 7 days of culture, cells were harvested, and purified populations of $\gamma\delta$ T cells were obtained using a human anti-TCR $\gamma\delta$ (11F2, mouse IgG1) MicroBead Kit (Miltenyi Biotec). Cell viability was determined using trypan blue exclusion.

Abs and reagents

To isolate the population of colon with cancer-initiating ability, disaggregated sphere cells were analyzed for the expression of epithelial specific Ag (ESA-FITC, clone B29.1; Biomed) and CD133 using CD133/2 (293C3-PE; Miltenyi Biotec). Jurkat, primary adherent colon cancer, and human cord blood CD34-enriched cells were used as negative and positive controls, respectively.

The following unconjugated, FITC-, PE-, PE-Cy5- or allophycocyanin-conjugated mAbs were obtained from BD Biosciences: anti-TCR V δ 2; anti-NKG2D; anti-Fas Ligand; anti major histocompatibility complex class I-related molecules (MIC) A/B; and anti-TNF- α . The following purified mAbs were also used: anti-CD3 (blocking, MEM-57), anti-HLA class I monomorphic (MEM-147), and anti-HLA-E (MEM-E/6), all from Professor Vaclav Horejsi (Institute of Molecular Genetics, Prague, Czech Republic); purified anti-TCR pan $\gamma\delta$ (IMMU510, a gift from Dr. Marc Bonneville, Institut de Biologie, Nantes, France); anti-TRAIL-R1 (HS101, mouse IgG1; Alexis), anti-TRAIL-R2 (HS202, mouse IgG1, Alexis), and anti-perforin (δ G9; Alexis).

Cells were stained according to manufacturers' recommendations and analyzed using a FACSCalibur supported with CellQuest acquisition and data analysis software (BD Biosciences). The lymphocytes were gated for forward/side scatter.

Proliferation, ELISA, and N $^{\alpha}$ -carbobenzoxy-L-lysine-thiobenzyl (BLT) esterase assays

V γ 9V δ 2 T cells were cultured at 37°C, in 5% CO₂, at 10⁶/ml in 96-well flat-bottom plates (0.1 ml/well), with an equal number of colon CSCs that had been pretreated or not with zoledronate for 24 h, and 20 U/ml IL-2. In some experiments, colon CSCs were pulsed with zoledronate for 2 h and then extensively washed before culture. As a positive control for expansion, V γ 9V δ 2 T cells were cultured with isopentenyl pyrophosphate (IPP; Sigma-Aldrich; 10⁻⁵ M final concentration), 20 U/ml IL-2, and an equal number of irradiated (30 Gy from a cesium source) allogeneic human monocytes/macrophages (CD14⁺ cells; Refs. 28 and 29). Seven days later, cells were collected, and the percentage of V γ 9V δ 2 cells within the CD3⁺ population was assessed by FACS. The absolute number of V γ 9V δ 2 T cells present in each culture was calculated according to the formula (% V γ 9V δ 2 T cells \times total number of viable cells)/100. The V γ 9V δ 2 T cell expansion factor (EF) was then calculated by dividing the absolute number of V γ 9V δ 2 T cells in stimulated cultures by the number of V γ 9V δ 2 T cells cultured in the absence of IPP or CSCs (28). In some experiments, V γ 9V δ 2 T cells were labeled with CFSE (Molecular Probes) before culture in vitro.

IFN- γ , TNF- α , and TRAIL levels were assessed in the 48-h culture supernatants by two-mAb sandwich ELISA following the manufacturer's recommendations (R&D Systems; Ref. 27). BLT esterase levels in 24-h culture supernatants were determined by the BLT esterase assay. Briefly, 20 μ l of culture supernatant were incubated with 350 nM BLT, 350 nM 5–5'-dithiobis-(2-nitrobenzoic acid), and 0.01% Triton X-100 (all from Sigma-Aldrich). After incubation for 30 min at 37°C, optical density was measured at 405 nm (27, 28).

Cytotoxic assay and flow cytometry

Purified $\gamma\delta$ T cells were resuspended at the final concentrations of 1.5×10^6 , 3×10^6 , and 6×10^6 cells/ml, and 100 μ l were then added to round-bottom polystyrene tubes together with colon cancer sphere cells (100 μ l), to obtain E:T ratios of 5:1, 10:1, and 20:1. Treatment with CFSE was performed as follows. Fifty microliters of CFSE were added to 1 ml of target sphere cell suspension (5×10^5 cells/ml) in PBS to obtain the final concentration of 2.5 μ M CFSE. The cells were incubated for 10 min at 37°C and gently mixed every 5 min. At the end of

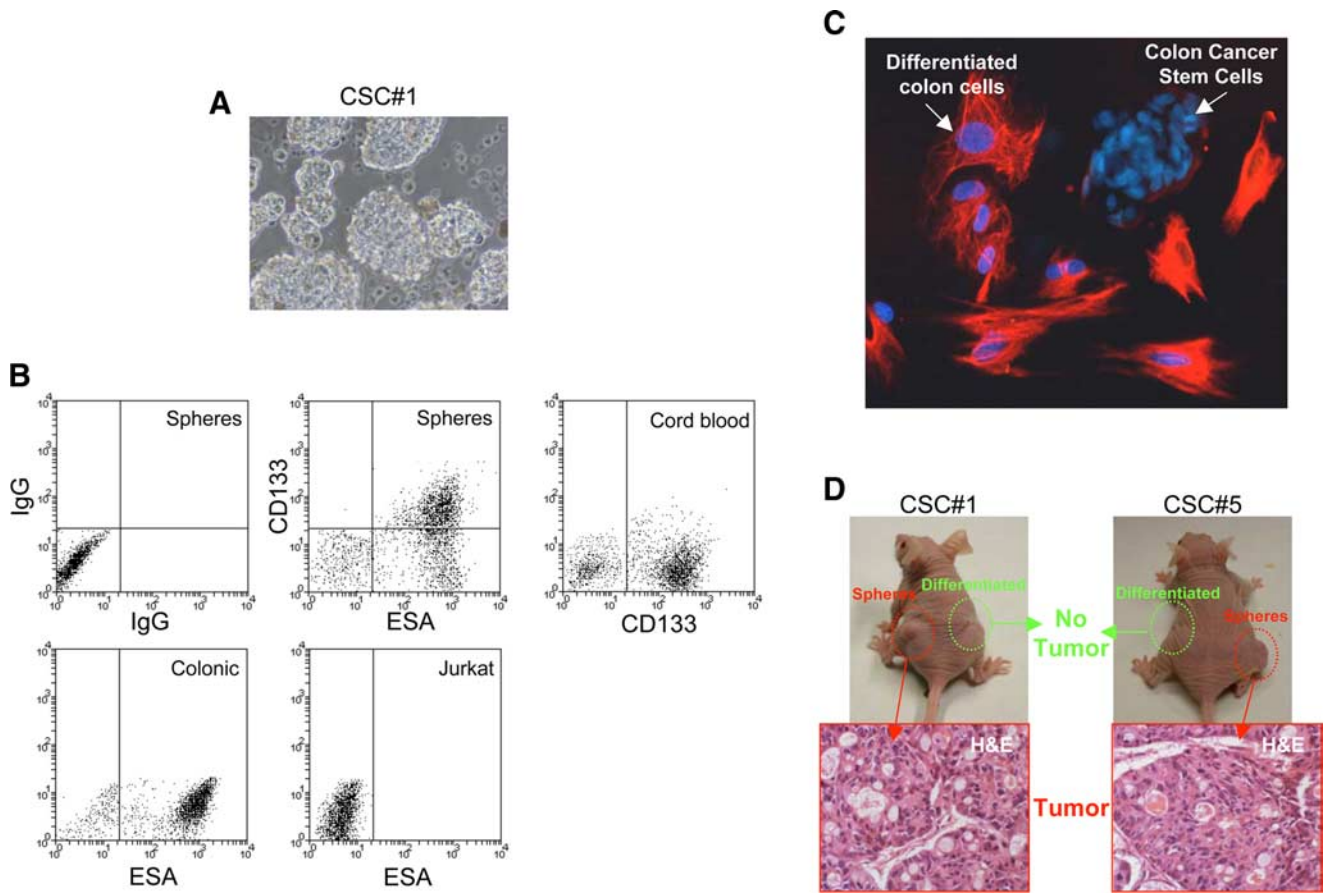


FIGURE 1. Colon CSCs express CD133 and ESA markers and display a gradual acquisition of colon epithelial markers during in vitro differentiation. *A*, Representative phase contrast microscopy analysis of colorectal cancer spheres. *B*, Flow cytometric profiles of disaggregated colon sphere, Jurkat, and human cord blood CD34⁺ cells for isotype-matched controls, CD133 and ESA. *C*, Representative scanning confocal microscopy analysis of villin on colorectal cancer spheres after 6 days of differentiation in the presence of 10% FBS. *D*, s.c. tumor growth in nude mice 10 wk after injection of 2000 disaggregated cells from colon cancer spheres (*top*). H&E staining analyses on paraffin-embedded sections of cancer xenografts. Nuclei are revealed by hematoxylin staining (blue). One representative experiment of two performed with cells from different donors is shown.

incubation, 1 ml of 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.

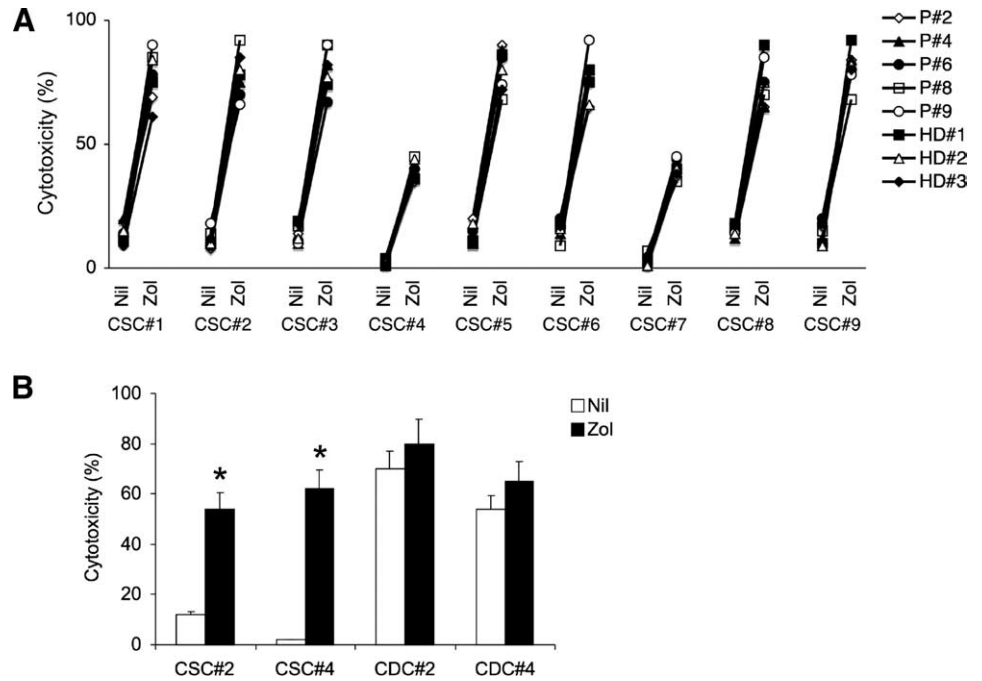
Control tubes containing only labeled target cells and effector cells were also prepared to establish background levels of cells death. Tubes were gently mixed, centrifuged at 300 × *g* for 2 min and incubated at 37°C in 5% CO₂ for 4 h. At the end of the incubation period, the tubes were placed

Table I. Cytotoxic activity of Vγ9Vδ2 T lymphocytes from various origin against colon cancer stem cells, colon cancer differentiated cells, and normal colon epithelial cells^a

	Vγ9Vδ2 T Cell Lines and Clone							
	P#2	P#4	P#6	P#8	P#9	HD#1	HD#2	HD#3
CSC#1	19 ± 4	20 ± 3	10 ± 3	12 ± 1	14 ± 2	12 ± 3	15 ± 4	9 ± 2
CSC#2	8 ± 1	12 ± 2	9 ± 1	14 ± 2	18 ± 2	10 ± 1	10 ± 1	12 ± 2
CSC#3	14 ± 2	10 ± 1	11 ± 2	17 ± 3	10 ± 2	10 ± 3	12 ± 1	18 ± 4
CSC#4	3 ± 1	4 ± 1	<1	<1	2 ± 2	4 ± 1	2 ± 1	<1
CSC#5	20 ± 3	10 ± 4	15 ± 2	11 ± 1	10 ± 1	10 ± 4	18 ± 3	12 ± 1
CSC#6	16 ± 2	14 ± 2	20 ± 2	9 ± 2	15 ± 3	18 ± 1	16 ± 2	17 ± 4
CSC#7	<1	2 ± 1	<1	7 ± 2	4 ± 3	3 ± 2	<1	5 ± 1
CSC#8	15 ± 2	12 ± 2	15 ± 2	16 ± 3	14 ± 1	18 ± 3	14 ± 3	18 ± 2
CSC#9	17 ± 2	12 ± 4	18 ± 2	15 ± 2	20 ± 3	10 ± 3	9 ± 2	20 ± 5
DLD-1	54 ± 12	48 ± 9	71 ± 16	84 ± 10	52 ± 12	64 ± 13	72 ± 14	81 ± 12
SW620	63 ± 8	52 ± 10	60 ± 10	80 ± 20	64 ± 12	70 ± 13	81 ± 15	70 ± 14
SW403	70 ± 14	58 ± 14	58 ± 6	56 ± 18	65 ± 8	54 ± 10	62 ± 8	80 ± 15
CL-241	<1	1 ± 1	<1	<1	2 ± 1	<1	3 ± 1	<1

^a Eight different Vγ9Vδ2 T cell lines were obtained from three healthy donors (HD#1, #2, and #3) and five different patients affected by colon cancer (P#2, #4, #6, #8, and #9). These populations were tested for cytotoxic activity against colon cancer sphere cells from nine different patients (CSC#1–CSC#9), differentiated colon cancer cell lines (DLD-1, SW620, and SW403), and normal colon epithelial cells (CL-241) at an E:T ratio of 20:1. In bold are indicated two CSC lines resistant to killing by all tested Vγ9Vδ2 T cell lines. Data are means ± SD of six different experiments, each performed in triplicate.

FIGURE 2. Zoledronate sensitizes colon CSCs to V γ 9V δ 2 cell-mediated cytotoxicity. **A**, Cytotoxicity percentage of eight different V γ 9V δ 2 T cell lines obtained from three healthy donors (HD#1, #2, and #3) and five different patients affected by colon cancer (P#2, #4, #6, #8, and #9), against colon cancer sphere cells from nine different patients (CSC#1–CSC#9) at an E:T ratio of 20:1. Colon CSC targets were pretreated or not (Nil) with zoledronate for 24 h (Zol). **B**, Cytotoxicity of one V γ 9V δ 2 T cell line against colon CSCs and differentiated colon cancer cells (CDC) obtained from patients 2 and 9, that had been pretreated or not with zoledronate for 23 h. *, $p < 0.001$ when compared with CSCs treated with medium alone.



on ice, and 20 μ l of propidium iodide (PI) (1 μ g/ml) were added to each tube for 10–15 min in ice. Finally, 100 μ l of complete medium were added before acquisition on a 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⁻), according to Ref. 30.

Blocking studies

Blocking agents were used to evaluate the mechanisms of $\gamma\delta$ T cell-mediated recognition and cytotoxicity of CSCs. To evaluate the contribution of zoledronate-induced accumulation of mevalonate metabolites to kill target cells, tumor cells were treated with mevastatin (25 μ M for 2 h; Sigma-Aldrich) a selective upstream inhibitor of the mevalonate pathway. Target cells were then incubated with 200 nM zoledronate for 24 h. After this incubation period, target cells were washed, and $\gamma\delta$ T cells added at a 20:1 ratio, in the presence of 25 μ M mevastatin, to maintain a constant concentration of this drug during incubation because its effect is rapidly reversible (16).

To inhibit perforin-mediated cytotoxicity, $\gamma\delta$ T cells were incubated with concanamycin A (CMA; Sigma-Aldrich) at 15 nM for 30 min at 37°C before coculture without further washing (24).

To block the relevant cytotoxic pathways, specific or isotype-control mAbs were used at 10 μ g/ml final concentration just before cocultivation assay.

Histochemistry, immunofluorescence, and confocal microscope analysis

Histochemical analysis was assessed on 6- μ m paraffin-embedded sections of tumor xenografts (5). H&E staining was performed following the manufacturer's instruction. For immunofluorescence, differentiated colon cancer sphere cells monolayers were fixed with 2% paraformaldehyde for 20 min at 37°C, washed in PBS, and exposed for 1 h at 37°C to Abs against villin (C-19, sc-7672, goat polyclonal; Santa Cruz Biotechnology). Then, cells were treated with Rhodamine Red-conjugated anti-goat Abs (Molecular Probes) plus RNase (200 ng/ml) and counterstained using Toto-3 iodide (642/660; Molecular Probes).

Immunofluorescence was also performed on coculture of $\gamma\delta$ T cells and colon cancer disaggregated sphere cells (E:T, 20:1). Cells were centrifuged onto microscope slides, fixed, washed as previously described, and exposed overnight at 4°C to Abs specific for CD133/2 (AC141 mouse IgG1; Miltenyi Biotec). CD133-labeled cells were then exposed for 1 h at 37°C to Rhodamine Red-conjugated anti-mouse Ab (Molecular Probes). Successively, cells were fixed again as above described, treated with NH₄Cl (50 mM) to remove fluorescence background, incubated with specific Ab against TCR V δ 2 overnight at 4°C, washed twice in PBS, and exposed to Texas Red-conjugated anti-mouse Ab (Molecular Probes) as above. Fi-

nally, TUNEL reaction was assessed on CD133- and V δ 2TCR-labeled cells. Apoptosis was determined with an In Situ Cell Death Detection kit (Boehringer Mannheim) and DNA strand breaks were detected by green fluorescence. Positive control was performed by pretreating specimens with DNase I, whereas the negative control was represented by CD133⁺ and V δ 2TCR⁺ cells subjected to the same staining for TUNEL without TdT (data not shown). Confocal analysis was used to acquire fluorescence stainings.

Statistics

The two-tailed Student *t* test was used to compare significance of differences between groups. All values are expressed as mean \pm SD.

Results

Zoledronate sensitizes colon CSCs to V γ 9V δ 2 T cell killing

Our previous data suggest that colon cancer is characterized by a pool of differentiated cells that comprise the majority of the total tumor cells and a small population of cells expressing the surface marker CD133, which is responsible for tumor initiation and maintenance (5). For the purposes of this study, we successfully purified and propagated colon cancer spheres from surgical fragments of nine patients with colon carcinoma (Fig. 1A). These cancer sphere lines were identified through the expression of CD133 and the epithelial-specific Ag ESA (Fig. 1B). Colon cancer sphere cultures in the presence of serum displayed adherence to the culture dishes and subsequently differentiated into large, polygonal colon cells expressing colon epithelial markers, such as villin (Fig. 1C). Thus, these data suggest that colon cancer spheres maintained the ability to differentiate in vitro in enterocyte-like cells. More importantly, a low number of colon cancer spheres when injected s.c. into nude mice, differently from sphere-derived differentiated cells, retained the capacity to form a tumor (Fig. 1D, top) that closely resembled the human original tumor, mainly containing differentiated cells (Fig. 1D, bottom).

We then generated polyclonal V γ 9V δ 2 T cell lines after immunomagnetic sorting of V δ 2 cells from PBL from three healthy donors (allogeneic $\gamma\delta$ cells) and from five patients (autologous $\gamma\delta$ cells).

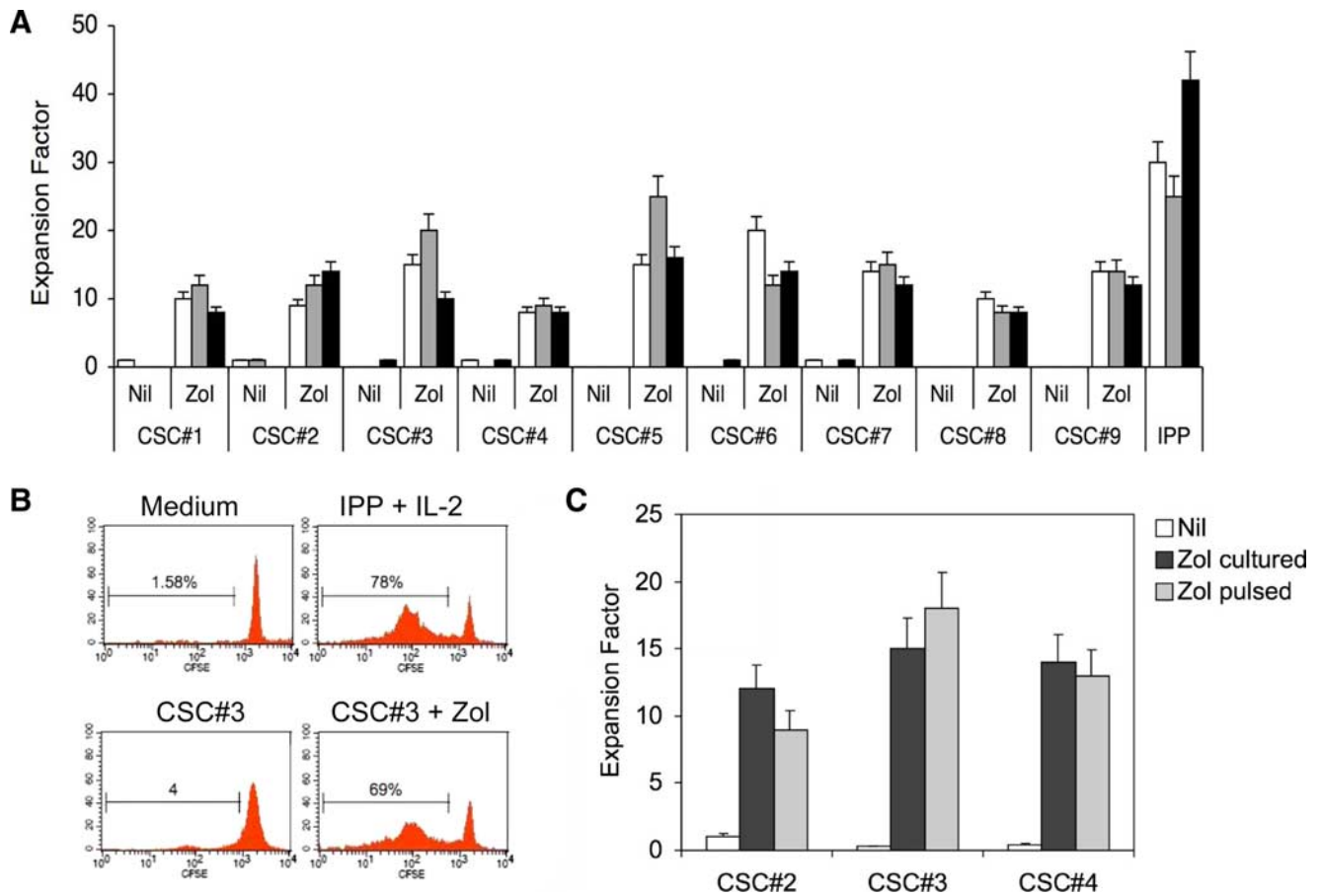


FIGURE 3. Zoledronate-treated colon CSCs stimulate expansion of V γ 9V δ 2 T cells. **A**, Colon CSCs were treated with zoledronate for 24 h (Zol) or left untreated (Nil) and were cocultured with three different allogeneic V γ 9V δ 2 T cell lines (HD#1, \square ; HD#2, \square ; HD#3, \blacksquare) in vitro for 7 days in the presence of 20 U/ml final concentration IL-2. At the end of the culture period, the V γ 9V δ 2 T cell EF was calculated as described in *Materials and Methods*. Data are means \pm SD of nine different experiments, each conducted in triplicate. **B**, Results of a representative experiment showing that CFSE-labeled V γ 9V δ 2 T cells proliferate upon culture with zoledronate-treated CSC#3 cell line. **C**, Three different colon CSCs (CSC#2, #3, and #4) were treated with zoledronate for 24 h (Zol cultured) or pulsed for 2 h with zoledronate (Zol pulsed) or left untreated (Nil) and were cocultured with a V γ 9V δ 2 T cell line in vitro for 7 days in the presence of 20 U/ml final concentration IL-2. At the end of the culture period, the V γ 9V δ 2 T cell EF was calculated as previously described.

The ability of V γ 9V δ 2 T cells to kill colon CSCs was assessed before and after treatment of the targets with zoledronate for 24 h. V γ 9V δ 2 T cells from colon cancer patients and healthy donors were able to exert low, yet significant cytotoxicity on seven of the nine colon cancer sphere cells, ranging from 9 to 15% at an E:T ratio of 20:1 (Table I). Two colon CSC lines, CSC#4 and CSC#7, were resistant to V γ 9V δ 2 T cell killing, even when an E:T ratio of 50:1 was used. The poor cytotoxic activity toward CSC was not an intrinsic feature of the $\gamma\delta$ T cells, because the differentiated colon cancer DLD-1, SW620, and SW403 cell lines were recognized and efficiently killed by V γ 9V δ 2 T cell lines from colon cancer patients and healthy subjects. As expected, all the tested V γ 9V δ 2 T cell lines failed to kill the normal colon CCL-241 cell line (Table I).

V γ 9V δ 2 T cell cytotoxicity was also evaluated following target cell pretreatment with zoledronate for 24 h (Fig. 2A). Zoledronate pretreatment alone was sufficient to render colon CSCs almost completely susceptible to $\gamma\delta$ T cell killing, with high levels of cytotoxicity (>60%) achievable in seven of nine colon CSCs, at an E:T ratio of only 20:1. Even the colon cell lines CSC#4 and CSC#7, determined to be the targets resistant to V γ 9V δ 2 T cell killing (see Table I), were highly susceptible to zoledronate-induced sensitization, with increasing levels of cytotoxicity from 4–7% to 35–45%, at an E:T ratio of 20:1 (Fig. 2A). According to

data obtained with other bisphosphonates (16, 24, 31, 32), zoledronate also caused a limited increase (\sim 10%) in the cytotoxicity of the tested differentiated colon cancer cell lines: Fig. 2B shows representative results of the cytotoxic activity of V γ 9V δ 2 T cell line against two colon cancer stem cell lines (CSC#2 and CSC#4) and two differentiated colon cancer cell lines (CDC#2 and CDC#4) obtained from the same patients, #P2 and #P4.

Thus, zoledronate can be considered as an effective tool to selectively expand V γ 9V δ 2 T cells as well as to potentiate the susceptibility of target colon CSCs to V γ 9V δ 2 T cell-mediated cytotoxicity.

Zoledronate-sensitized colon CSCs expand V γ 9V δ 2 T cells and induce their production of cytokines and cytotoxic molecules

We next determined whether exposure to untreated or zoledronate-pretreated colon cancer sphere cell targets stimulated V γ 9V δ 2 T cells to expand, as well as to secrete cytokines (IFN- γ and TNF- α) and cytotoxic and proapoptotic molecules, such as TRAIL and BLT esterase (which most likely measures granzyme A). In three different experiments with three allogeneic V γ 9V δ 2 T cell lines, all the nine tested, zoledronate-treated CSC cell lines were able to expand V γ 9V δ 2 T cells, upon coculture in vitro for 7 days in the presence of low doses (20 U/ml final concentration) IL-2 (Fig. 3A). None of the untreated CSCs caused significant expansion of

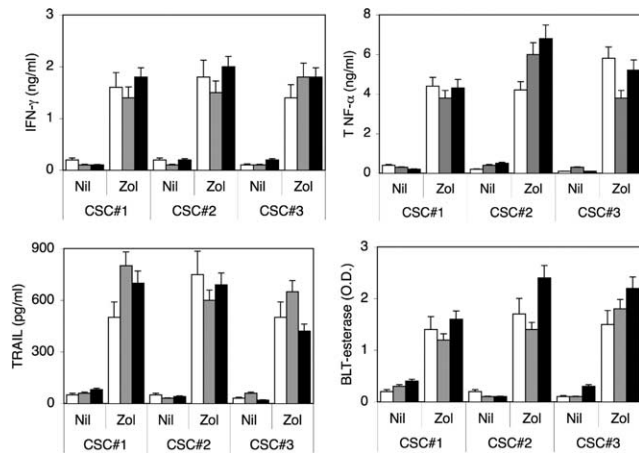


FIGURE 4. Zoledronate-treated colon CSCs trigger V γ 9V δ 2 T cells to produce cytokines and release cytotoxic molecules. Three different colon CSCs (CSC#1, #2 and #3) were treated with zoledronate for 24 h (Zol) or left untreated (Nil) and cocultured with 3 different allogeneic V γ 9V δ 2 T cell lines (HD#1, \square ; HD#2, \square ; HD#3, \blacksquare) in vitro for 7 days in the presence of 20 U/ml final concentration IL-2. IFN- γ (A), TNF- α (B), and TRAIL (C) production was assessed in 48-h supernatants, whereas BLT esterase release (D) was assessed in 24-h supernatants. Data are means \pm SD of experiments conducted in triplicate.

V γ 9V δ 2 T cells in the presence of IL-2 (Fig. 3A). In some experiments, the results of V γ 9V δ 2 T cell proliferation as measured by calculation of the increase in their absolute numbers (EF), were also confirmed by using CFSE dilution; typical results of a representative experiment using CFSE-labeled V γ 9V δ 2 T cell line and the colon CSC#4 line are shown in Fig. 3B.

To exclude the possibility that expansion of V γ 9V δ 2 T cells by zoledronate-treated colon CSCs might have been due to zoledronate itself released from the colon CSCs and passively transferred to the reactive V γ 9V δ 2 T cell during the 24-h culture, we pulsed CSCs with zoledronate for 2 h and then evaluated their ability to stimulate V γ 9V δ 2 T cells. As shown in Fig. 3C, 3 different colon CSC lines were fully able to induce expansion of V γ 9V δ 2 T cell either when cultured or when pulsed with zoledronate, thus excluding the possibility that any passively transferred zoledronate might be contributing to these findings. In similar experiments, we also tested the ability of zoledronate-treated or untreated CSCs to induce V γ 9V δ 2 T cells to secrete cytokines and cytotoxic molecules upon coculture in vitro for 24 h in the absence of any IL-2. In nine different experiments, both cytokines (IFN- γ and TNF- α) and cytotoxic molecules (TRAIL and BLT esterase) were abundantly secreted in supernatants only when V γ 9V δ 2 T cells were cocultured with zoledronate-pretreated colon CSCs, and this trend was evident regardless of the cell line used. Conversely, untreated colon cancer sphere cells induced very low, if any, production of cytokines and cytotoxic molecules. Fig. 4 shows representative data with three V γ 9V δ 2 T cell lines and three CSCs. Thus, once recognized, zoledronate leads to V γ 9V δ 2 T cells acquisition of effector functions that rely on the release of death mediators.

V γ 9V δ 2 T cell-mediated killing of zoledronate-sensitized CSC involves the TCR, NKG2D, and metabolites of the mevalonate pathway

It has been proposed that human $\gamma\delta$ T cells have several distinct pathways for antitumor immunity and killing of tumor cells functions can rely on secretion of proinflammatory cytokines and proapoptotic molecules or on cell contact-dependent lysis through a NK-like pathway or a TCR-dependent pathway. We found (Fig. 5)

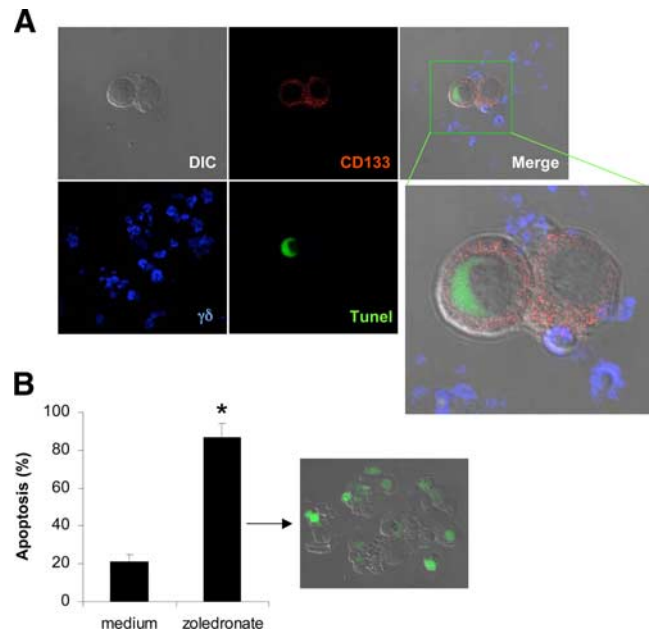


FIGURE 5. Colon CSCs treated with zoledronate are sensitized to apoptosis by V γ 9V δ 2 T cells. A, Scanning confocal microscopy analysis of DIC (upper left), CD133 (upper middle, red staining), V γ 9V δ 2 T cells (lower left, blue staining), TUNEL (lower middle, green staining) and merge (right) of coculture of zoledronate-treated colon CSCs with $\gamma\delta$ T cells at an E:T ratio of 20:1. One of six independent experiments, using six different colon CSC lines, each conducted with four different V γ 9V δ 2 T cell lines, has been shown. B, Apoptosis percentage of colon CSCs treated with medium alone or zoledronate and exposed to V γ 9V δ 2 T cells (E:T 20:1). Data are means \pm SD of six different experiments. A representative scanning confocal microscopy analysis performed as in A of this experiment is shown (green staining). *, $p < 0.001$ when compared with cells treated with medium.

that V γ 9V δ 2 T cells bind to colon CSCs leading to apoptosis induction, with a mean of 80% of apoptotic colon CSCs being detected in six different experiments, each conducted with three different V γ 9V δ 2 T cell lines. This finding suggests that besides the reported indirect mechanisms, V γ 9V δ 2 T cells can affect colon CSC susceptibility to death through a direct mechanism.

We assessed the mechanisms responsible for recognition of colon CSCs by V γ 9V δ 2 T cells, by individually blocking TCR or NKG2D receptors. Cytotoxicity of all zoledronate-pretreated colon CSC lines by two different allogeneic V γ 9V δ 2 T cell lines was inhibited at the greatest extent by anti-CD3 and anti-pan $\gamma\delta$ TCR Abs, indicating that cytotoxicity by V γ 9V δ 2 T cells is TCR-mediated. Addition of anti-HLA class I or anti-HLA-E Abs did not cause significant inhibition of cytotoxicity. Fig. 6A shows representative data with two V γ 9V δ 2 T cell lines and two colon CSC lines, CSC#2 and CSC#4. NKG2D seemed to play a lesser role in V γ 9V δ 2 T cells recognition and killing of zoledronate-sensitized colon CSCs, largely dependent on expression of NKG2D ligands by target cells. Thus, when target colon CSCs expressed some NKG2D ligands, as did the CSC#4 line (Fig. 6B), anti-NKG2D Ab significantly inhibited cytotoxicity, whereas no inhibition occurred if the target cells did not express such NKG2D ligands, as with the CSC#2 cell line.

In addition, V γ 9V δ 2 T cell recognition of zoledronate-sensitized targets was assessed in the presence of mevastatin, which inhibits 3-hydroxy-3-methylglutaryl-CoA and prevents zoledronate-mediated accumulation of IPP (33, 34). Mevastatin was found to inhibit killing of all tested zoledronate-pretreated colon

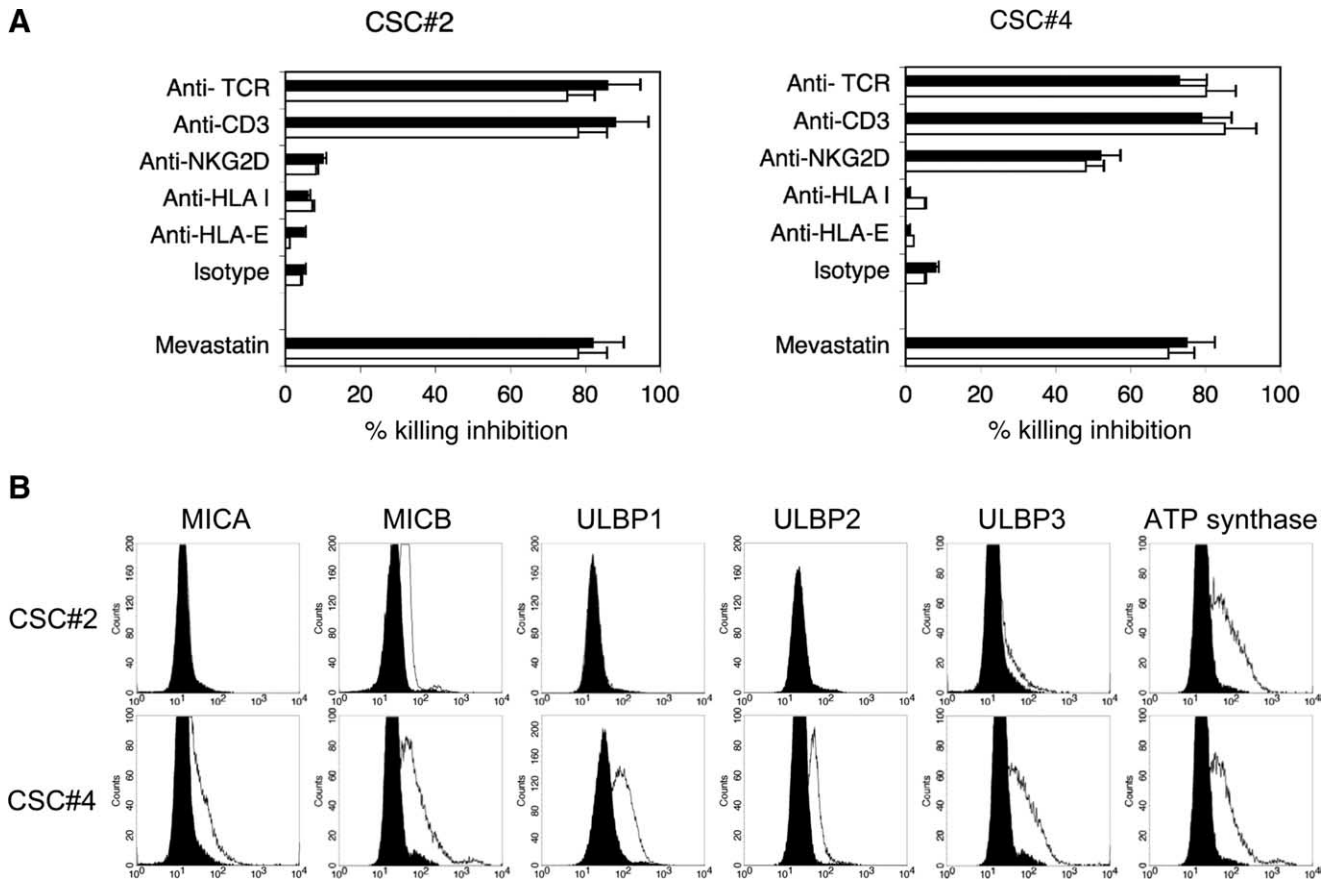


FIGURE 6. Modulation of the cytotoxic activity of V γ 9V δ 2 T cells by blocking the TCR or NKG2D interactions. **A**, Two different V γ 9V δ 2 T cell lines obtained from healthy donors (HD#1, \square ; HD#3, \blacksquare) were cultured with two zoledronate-treated colon CSCs (CSC#2 and CSC#4) at an E:T ratio of 20:1, in the presence of blocking Abs to the $\gamma\delta$ TCR, CD3, NKG2D, HLA class I and HLA-E molecules, or in the presence of mevastatin. Data are mean \pm S.D. of experiments conducted in triplicate. Percent inhibition with anti-TCR and -CD3 Abs and with mevastatin were significantly different than values in all other groups ($p < 0.001$), whereas percent inhibition killing of the CSC#4 line with anti-NKG2D Ab was significantly different than values with isotype control Ab ($p < 0.02$). **B**, Expression of the NKG2D ligands MICA, MICB, ULBP1, ULBP2, and ULBP3 and of ATP synthase by the CSC#2 and CSC#4 lines. The filled histograms represent isotype controls staining and the open ones are specific stainings.

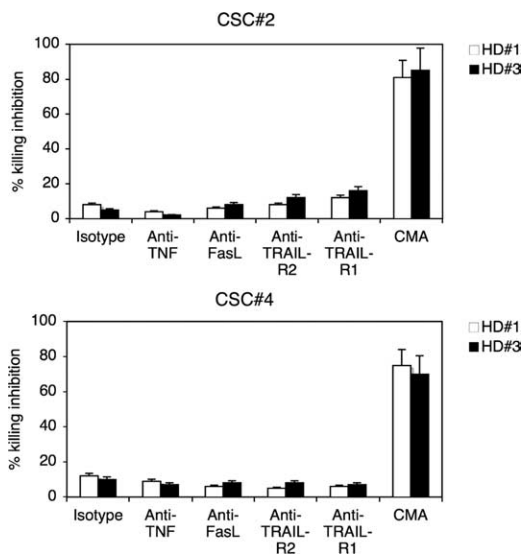


FIGURE 7. Modulation of the cytotoxic activity of V γ 9V δ 2 T cells by blocking the TCR or NKG2D interactions. Two different V γ 9V δ 2 T cell lines obtained from healthy donors (HD#1, \square ; HD#3, \blacksquare) were cultured with two zoledronate-treated colon CSCs (CSC#2 and CSC#4) at an E:T ratio of 20:1, in the presence of blocking Abs to TNF- α , FasL, TRAIL receptors R1 or R2, or CMA. Data are means \pm SD of experiments conducted in triplicate. Percent inhibition with CMA was significantly different than values in all other groups ($p < 0.001$).

CSC lines by two different allogeneic V γ 9V δ 2 T cell lines (Fig. 6A), thus indicating that production of mevalonate metabolites, most likely IPP, is required for colon CSC recognition by V γ 9V δ 2 T cells.

V γ 9V δ 2 T cells kill zoledronate-sensitized CSC targets via distinct mechanisms

To further elucidate the mechanisms responsible for killing of colon CSCs upon their interaction with V γ 9V δ 2 T cells, we individually inhibited the granule exocytosis, TNF- α , TRAIL-, and FasL-mediated pathways. Killing-inhibition experiments using CMA revealed that V γ 9V δ 2 T cell cytotoxicity of zoledronate-pretreated colon CSC targets was almost exclusively mediated by the perforin pathway (means of 80–84% inhibition using CMA), whereas Abs against TRAIL receptors 1 and 2, TNF- α , and FasL were unable to inhibit cytotoxicity in zoledronate sensitized targets. Fig. 7 shows representative data with two V γ 9V δ 2 T cell lines and the two colon CSCs lines, CSC#2 and CSC#4.

Discussion

The refractoriness to drug treatments of CSCs has been attributed to a variety of aspects including the action of chemotherapeutic drugs which target dividing cells and are consequently unable to kill the presumed slow-cycling CSCs, and the expression of high levels of anti-apoptotic proteins and ABC (ATP Binding Cassette)

transporters particularly effective at pumping out the drugs. Moreover, in the context of colon carcinoma, we have recently shown that drug resistance is due to the autocrine production of IL-4 and that the use of IL-4 blockers is sufficient to overcome this resistance (5). Apoptosis inhibition by IL-4, found in our work, is in accordance with the findings of several other studies on cancer (35–39) but is in contrast with reports where IL-4 has been shown to increase cell death (40, 41). However, clinical trials of IL-4 therapy have been often disappointing with scarce antitumor effects or even with cancer cell expansion (42, 43).

Here, we describe an immune response-based approach in the population of colon CSCs highly resistant to the conventional chemotherapeutic drugs and to putative innovative therapies such as those based on the use of TRAIL. Particularly, we show that zoledronate is able to sensitize malignant colon CSCs targets to V γ 9V δ 2 T cell cytotoxicity. Although V γ 9V δ 2s are able to recognize and kill many different differentiated tumors cells, either spontaneously or after treatment with different bisphosphonates, including zoledronate (22–25, 32), to our knowledge, this is the first report describing the recognition and efficient killing of colon CSCs by V γ 9V δ 2 T cells. Because normal cells are not killed by V γ 9V δ 2 T cells (see also Table I), the lytic potential of V γ 9V δ 2 T cells appears selective for transformed epithelial cells, as it is for transformed hemopoietic cells, such as leukemia, myeloma, and lymphoma cells (16, 22, 44, 45).

Human V γ 9V δ 2 T cells recognize small nonpeptidic phosphorylated compounds, referred to as phosphoantigens, which are metabolites of isoprenoid-biosynthetic pathways (18, 46, 47). Furthermore, the expression of IPP has been shown to correlate with the susceptibility of some tumor cell lines to V γ 9V δ 2 T cell cytotoxicity (22, 48–51). Data reported in this study obtained through stimulation or inhibition of IPP biosynthesis by zoledronate and mevastatine, respectively, suggest that colon CSCs recognition might also correlate with increased expression/production of mevalonate metabolites. Consistent with this assumption are the findings that 1) pretreatment of tumor cells by zoledronate enhances colon CSCs killing without modifying the tumor cell phenotype (data not shown), suggesting that zoledronate treatment leads to intracellular accumulation of IPP within the cell, favoring recognition of the tumor cells by V γ 9V δ 2 T cells, and 2) inhibition of the limiting enzyme 3-hydroxy-3-methylglutaryl-CoA reductase (33, 34) by mevastatin, significantly reduces the killing of colon CSCs by V γ 9V δ 2 T cells.

V γ 9V δ 2 T cells also recognize an F1-ATPase-related structure and one of its ligands, a delipidated form of apolipoprotein A1 (52), that unlike in normal cells is ectopically expressed on the surface of hemopoietic tumors and renal cancer cell lines. ATP synthase expression can also be detected on colon CSCs (Fig. 6B) but currently available anti-ATP synthase Abs are not reliable for inhibition studies; therefore, further studies with better reagents are required to evaluate whether ATP synthase may play a role in recognition and killing by V γ 9V δ 2 T cells.

V γ 9V δ 2 T cell activity is tightly regulated by NK-like receptors for MHC class I ligands, and some previous studies have indicated the importance of NKG2D-MICA/B interactions for tumor cell recognition and cytotoxic activity by V γ 9V δ 2 T cells (53, 54). Our data clearly indicate that recognition and killing of colon CSCs are mainly TCR mediated. However, NKG2D receptor could deliver a costimulatory signal enhancing the lysis of those colon CSCs that express MICA/B. Accordingly, we show here that NKG2D contributed to colon CSCs cytotoxicity by V γ 9V δ 2 T cells, assuming the target express high levels of several NKG2D ligands, such as MICA and ULBP1, -2, and -3, as occurred for instance with the CSC#7 line (Fig. 6). Conversely, when the target cell line ex-

pressed low, if any, levels of NKG2D ligands, as occurred with the CSC#2 line (Fig. 6), cytotoxicity was exclusively mediated by the TCR.

V γ 9V δ 2 T cells, upon recognition, kill tumor targets via a number of mechanisms including death receptor/ligand interactions with TRAIL and FasL, and by release of perforin/granzymes or cytokines such as TNF- α . One or more of these pathways may be involved in the killing of colon CSCs. Although colon CSC lines evaluated in this study expressed DR4, DR5, and Fas, this expression did not initially translate into sensitivity to V γ 9V δ 2 T cell killing, as documented by the failure of specific blocking Abs to inhibit cytotoxicity. Regardless of the colon CSC line tested, the finding that CMA strongly inhibited cytotoxicity indicates that zoledronate-treated targets are almost exclusively killed by perforin release by V γ 9V δ 2 T cells, consistent with previous findings of perforin/granzyme-dominated killing (32, 55–58).

V γ 9V δ 2 T cells have been detected in the majority of colon cancer tumor-infiltrating lymphocyte populations, and the response of this T cell subpopulation to colon cancer cells suggests that a natural immune response mediated by these lymphocytes contributes to the immunosurveillance of these tumors. Such observations may foster the development of novel alternative or adjuvant therapies targeting V γ 9V δ 2 T cells for the treatment of tumor patients, including those affected by colon cancer (59).

This might be achieved for instance by stimulating V γ 9V δ 2 T cells in these patients through injection of zoledronate and IL-2, as recently performed in hematological malignancies (60) and in prostate cancer (27). Alternatively, it could be achieved by adoptive transfer of ex vivo expanded autologous V γ 9V δ 2 T cells derived from cancer patients (61, 62). These T cells could be obtained from patient blood, because PBL-derived V γ 9V δ 2 T cells exhibited a similar reactivity than tumor-infiltrating lymphocytes toward colon cancer cells (16), even if ex vivo expanded, tumor-infiltrating V γ 9V δ 2 T cells migrate toward primary renal tumor cells with higher efficiency than their peripheral blood expanded counterparts (63). Both the $\gamma\delta$ T cell transfer and the infusion of bisphosphonates have been proven to be well tolerated (27, 60–62). However, care should be taken in different types of malignancies, depending on the infiltrating $\gamma\delta$ subset. For instance, in lymphoid malignancies, an expanded population of V δ 1 cells producing IL-4 has been found, which correlates with good prognosis in a 1-year follow-up (64).

In conclusion, it can be speculated that the in vitro expansion of $\gamma\delta$ T cells and the subsequent infusion of these cells plus zoledronate in combination with other antitumor agents may be of significant clinical benefit in the treatment of colon cancer and probably also of other forms of cancer.

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Disclosures

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