Targeting Human γδ T Cells with Zoledronate and Interleukin-2 for Immunotherapy of Hormone-Refractory Prostate Cancer

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Abstract

The increasing evidence that γδ T cells have potent antitumor activity suggests their value in immunotherapy, particularly in areas of unmet need such as metastatic carcinoma. To this end, we initiated a phase I clinical trial in metastatic hormone-refractory prostate cancer to examine the feasibility and consequences of using the γδ T-cell agonist zoledronate, either alone or in combination with low-dose interleukin 2 (IL-2), to activate peripheral blood γδ cells. Nine patients were enlisted to each arm. Neither treatment showed appreciable toxicity. Most patients were treated with zoledronate + IL-2, but conversely only two treated with zoledronate displayed a significant long-term shift of peripheral γδ cells toward an activated effector-memory–like state (T_{EM}), producing IFN-γ and perforin. These patients also maintained serum levels of tumor necrosis factor–related apoptosis inducing ligand (TRAIL), consistent with a parallel microarray analysis showing that TRAIL is produced by γδ cells activated via the T-cell receptor and IL-2. Moreover, the numbers of T_{EM} γδ cells showed a statistically significant correlation with declining prostate-specific antigen levels and objective clinical outcomes that comprised three instances of partial remission and five of stable disease. By contrast, most patients treated only with zoledronate failed to sustain either γδ cell numbers or serum TRAIL, and showed progressive clinical deterioration. Thus, zoledronate + IL-2 represents a novel, safe, and feasible approach to induce immunologic and clinical responses in patients with metastatic carcinomas, potentially providing a substantially increased window for specific approaches to be administered. Moreover, γδ cell phenotypes and possibly serum TRAIL may constitute novel biomarkers of prognosis upon therapy with zoledronate + IL-2 in metastatic carcinoma. [Cancer Res 2007;67(15):7450–7]

Introduction

Prostate cancer is the most common cancer diagnosis and the second leading cause of cancer-related deaths in men (1). Although 10-year cancer-specific survival rates are ~95% to 97% for radically prostatectomized patients with localized prostate cancer, up to one third such individuals experience disease recurrence (2, 3). In a median of 8 years after relapse, these patients will suffer from metastatic disease leading to death within another 2 to 5 years, independently of the applied treatment regimen (4). Advanced, recurrent, and metastatic tumors treated by androgen deprivation convert to androgen-independent growth within a few years and patients with metastatic hormone-refractory prostate cancer (HRPC) have a median survival of only 16 months. Treatment options are limited to aggressive chemotherapy with survival benefits of 2 to 3 months (5, 6). This lack of effective therapies for HRPC has fueled an intensive search for novel modalities, including immunotherapy (5–8).

There is good evidence that tumors can naturally be controlled by the immune system (9), and most immunotherapy strategies aim to induce adaptive, tumor-specific responses of B cells and MHC-restricted αβ T cells, particularly CD8 T cells. Nonetheless, despite major advances in this area, and the introduction of vaccine-based strategies, durable responses are rare and active immunotherapy is not yet an established modality. Tumor immunoevasion mechanisms are common and include the down-regulation of tumor-associated antigens, of MHC, and of costimulatory molecules (9, 10). By contrast to αβ T cells, γδ T cells are not MHC restricted and show less dependence on costimulators such as CD28. Moreover, γδ T cells are a natural component of resistance to cutaneous carcinogenesis in mice (11), and in humans display potent MHC-unrestricted cytotoxic activity *in vitro* against various tumors, including prostate cancer cell lines (12, 13). Indeed, human Vγ9Vδ2 T cells expanded *ex vivo* and then adoptively transferred to severe combined immunodeficient mice xenografted with tumor cells showed efficacy against B-cell lymphoma; melanoma; and renal, pancreatic, and nasopharyngeal carcinoma (14). Building on this, the potential of human γδ T cells for tumor immunotherapy has been directly analyzed and was recently expertly reviewed (15, 16).

Among γδ T cells, human peripheral blood Vγ9Vδ2 T cells recognize low molecular mass nonpeptide ligands (17–20). Such "phosphoantigens" comprise isoprenoid pathway metabolites, as (*E*)-4-hydroxy-3-methyl-but-2-enyl PPi (HMB-PP) derived from bacteria and protozoa (19) and isopentenyl PPi (IPP) derived from host cells (20). Accordingly, pharmacologic agents, such as aminobisphosphonates, that cause an accumulation of such metabolites (21), sensitize tumor cells to Vγ9Vδ2 T cells. Recognition may then be sustained by the tumor cell expression of NKG2D ligands, such as MICA and MICB, and of an ATPase that binds the Vγ9Vδ2 T-cell receptor (TCR; ref. 22), and that unlike in normal cells is ectopically expressed on the tumor cell surface.

Note: Supplementary data for this article are available at Cancer Research Online (http://cancerres.aacrjournals.org/).

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Like other T cells, Vγ9Vδ2 lymphocytes comprise distinct populations distinguishable on the basis of surface markers, effector functions, and trafficking properties. Naive (T_{naive},CD45RA$^+$CD27$^+$) and central memory (T_{CM}, CD45RA$^-$CD27$^+$) cells home to secondary lymphoid organs and lack immediate effector functions, whereas the so-called effector-memory (T_{EM}, CD45RA$^-$CD27$^-$) and terminally differentiated (T_{EMRA}, CD45RA$^+$CD27$^-$) cells home to sites of inflammation where they display immediate effector functions such as cytokine production and cytotoxicity (23). Such functions are attractive weapons for immunotherapy strategies, and there are promising results from recent, relatively small-scale applications of Vγ9Vδ2 T cells to hematologic (24) and solid-tissue malignancies, including prostate cancer (25) and advanced renal cell carcinoma (26). Assessing that aminobisphosphonates such as zoledronate are already widely used in the clinic, we have conducted a phase I clinical trial in metastatic HRPC to determine the safety, feasibility, and response induced by activating Vγ9Vδ2 T cells *in vivo* using zoledronate either alone or in combination with low-dose IL-2. Although there has been a promising application of pamidronate and IL-2 to prescreened multiple myeloma and non–Hodgkin's lymphoma patients (24), there has been only one reported, small-scale application of zoledronate to solid tumors (25), and the consequences of a selective activation of $\gamma\delta$ T cells *in vivo* were unknown before the current study. In this regard, data presented here support the proposal that zoledronate + IL-2 may prove a novel, safe, feasible, and efficacious means to extend the life span of those with late stage metastatic carcinoma, and thereby to increase the window of the patients' availability for other, more tumor-specific molecular approaches.

Materials and Methods

Study design. Treatment of patients according to an institutional review board–approved protocol was done following written informed consent from each subject or subject's guardian. The study was designed only for metastatic HRPC, and 18 patients [age range 61–78 years (median 68 years)] pretreated with androgen deprivation and radiotherapy and/or chemotherapy were enrolled. Only patients with an expected life span of ~3 months, with the presence of bone metastasis and a Kernovsky index >60%, were included. All patients had a prostate-specific antigen (PSA) increase of ≥20% within 2 months. The median pretreatment PSA doubling time, calculated as described in ref. (27) was 4.1 months (range 4.3–3.9 months, respectively), and the median time to progression was 10.6 months (95% CI, 7.8–13.2 months). No statistically significant difference in either PSA doubling time or time to progression was found between patients enrolled to receive zoledronate and zoledronate + IL-2. With the exception of patients 1B and 2B, all other patients received docetaxel

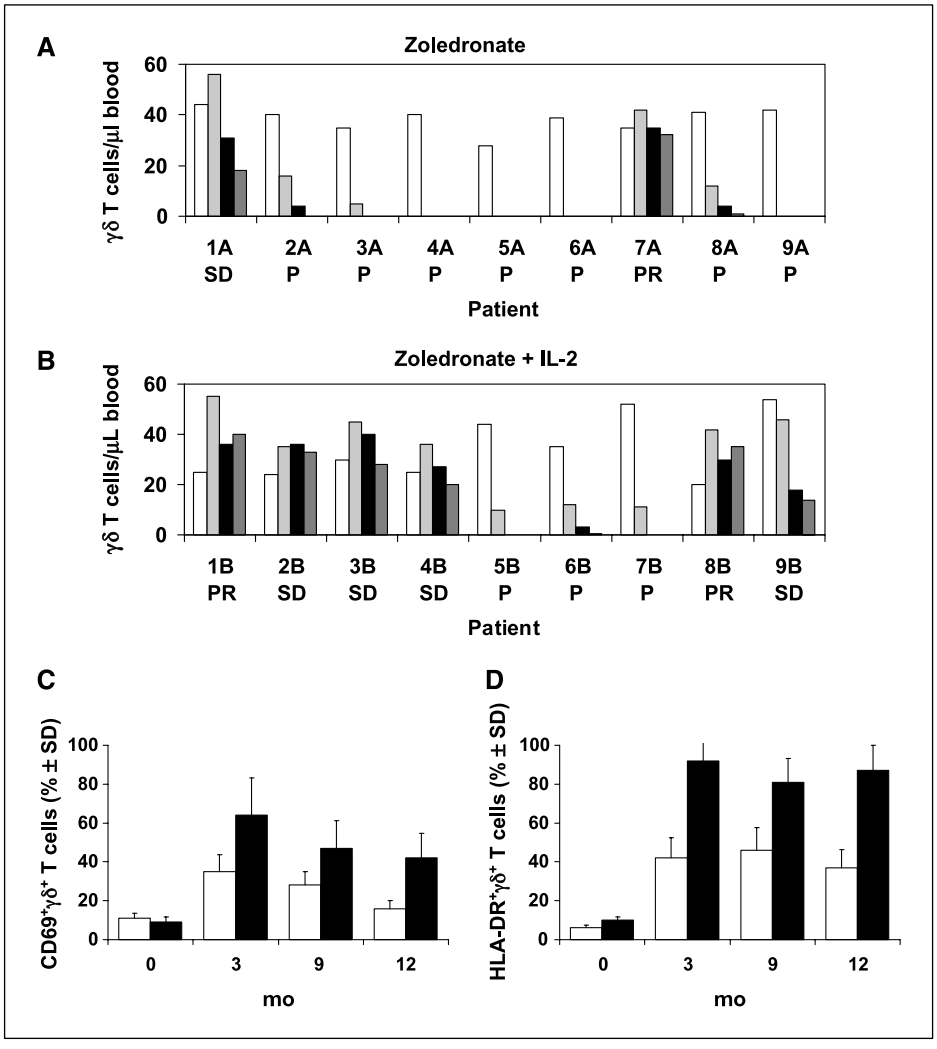

Figure 1. $\gamma\delta$ T-cell numbers and activation markers in patients treated with zoledronate or zoledronate + IL-2. PBMC were obtained from patients treated with zoledronate alone (*A*) or with zoledronate + IL-2 (*B*), before starting treatment (*white columns*) and 3 mo (*gray columns*), 9 mo (*black columns*), and 12 mo (*hatched columns*) after treatment. Where no data are shown, patients had already died. PBMC were double stained with anti-CD3 and anti-Vδ2 mAbs and the absolute number of Vγ9Vδ2 T cells was calculated. The *X*-axis labeling indicates individual patients and their clinical status (*P,* progression; *SD,* stable disease; *PR,* partial remission). PBMC were stained with anti-CD3, anti-Vδ2, anti-CD69, and anti–HLA-DR mAbs. *Columns,* mean percentage of $\gamma\delta$ T cells expressing CD69 (*C*) and HLA-DR (*D*) molecules in patients treated with zoledronate alone (*empty columns*) or with zoledronate + IL-2 (*filled columns*); *bars,* SD.

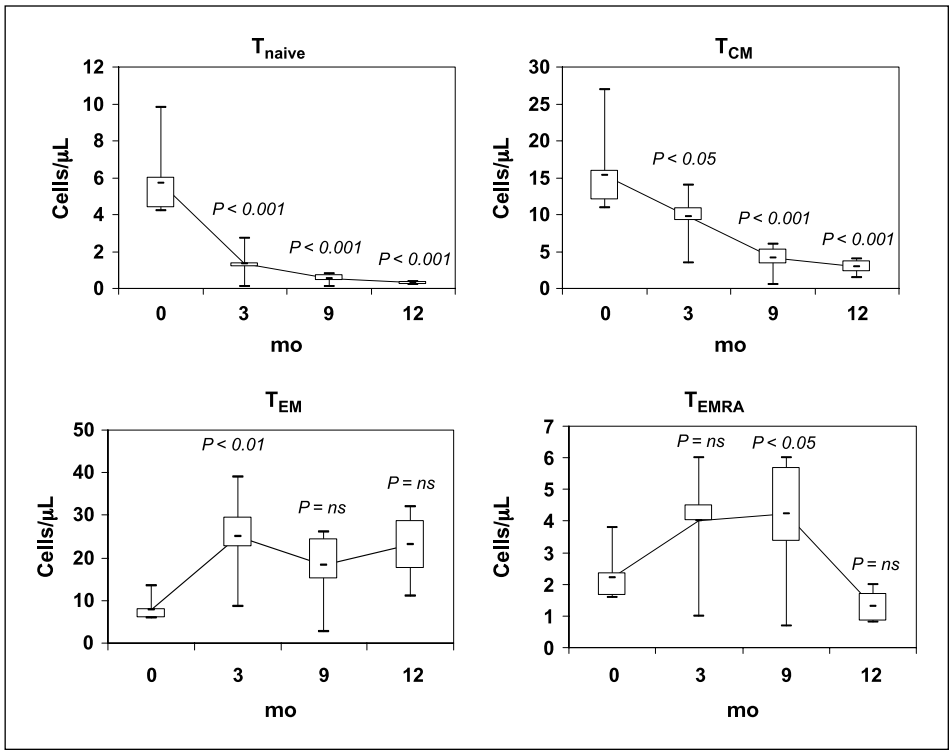

Figure 2. Distribution of γδ T-cell subsets in patients treated with zoledronate + IL-2. PBMC were obtained from seven patients treated with zoledronate + IL-2, before starting treatment (month 0), and 3, 9, and 12 mo after treatment. After four-color staining of PBMC with anti-CD3, anti-Vδ2, anti-CD45RA, and anti-CD27 mAbs, the absolute number of Vγ9Vδ2 T_{naive} ($CD45RA^+CD27^+$), T_{CM} ($CD45RA^-CD27^+$), T_{EM} ($CD45RA^-CD27^-$), and T_{EMRA} ($CD45RA^+CD27^-$) cells was calculated. *Top, bottom, and line through the middle of the boxes,* 75th, 25th, and 50th percentiles. *Lines that extend from the boxes,* the highest and the lowest values from each subgroup. *Lines within the boxes,* median values.

36 mg/m^2 i.v. over 15 to 30 min weekly for 6 consecutive weeks, on an 8-week cycle. All the patients had received four to five cycles docetaxel before being enrolled in this study. Patients were excluded if they were receiving any chemotherapy, radiation therapy, or bisphosphonates over a period 6 weeks before study entry; if they had severe cardiovascular disease, refractory hypertension, symptomatic coronary artery disease; a serum creatinine of >3.0 mg/dL or a corrected (for albumin) serum calcium of <8.0 mg/dL; if they had CNS metastases; a history of autoimmunity, with serious intercurrent chronic or acute illnesses or concurrent second malignancy; or if they were being treated with steroids or other immunosuppressive agents. Treatment with antiandrogen was withdrawn at least 4 weeks before study entry, whereas continuation of gonadal androgen suppression was conducted on all patients on luteinizing hormone-releasing hormone analogues. The main characteristics of the patients are shown in Supplementary Table S1. The clinical status of patients was evaluated according to Response Evaluation Criteria in Solid Tumors (28).

Treatment schedule. Eighteen subjects with metastatic HRPC were enrolled and randomized to receive either zoledronate (cohort A) or zoledronate + IL-2 (cohort B). Zoledronate (Novartis) was administered by a 15 min 100 mL i.v. infusion of normal saline at a standard dose of 4 mg, every 21 days. IL-2 (Chiron, 0.6×10^6 IU) was administered s.c. immediately after each zoledronate administration. All patients received 500 mg calcium supplement and 400 IU of vitamin D daily. This treatment schedule was done for 1 year.

Flow cytometry. Patients' blood samples were collected before (month 0) and at 3 months (6 days after the fourth administration of zoledronate or zoledronate/IL-2), 9 months (6 days after the 13th administration of zoledronate or zoledronate/IL-2), and 12 months (6 days after the 17th administration of zoledronate or zoledronate/IL-2) after the study began. Peripheral blood mononuclear cells (PBMC) were obtained by Ficoll-Hypaque centrifugation and stained with FITC-, phycoerythrin-, phycoerythrin-Cy5–, or allophycocyanin-conjugated antibodies against CD27, CD45RA, CD69, HLA-DR, CD3, CD56, CD4, CD8, TCR pan αβ, TCR pan γδ, TCR Vγ9, or TCR Vδ2 [all monoclonal antibodies (mAb) were from BD PharMingen]. Cells (10^5) from each sample were analyzed using a FACScalibur supported with CellQuest acquisition and data analysis software (Becton Dickinson). The lymphocytes were gated for forward/side scatter.

Analysis of Vγ9Vδ2 T cells. The medium used throughout was complete RPMI 1640 (Life Technologies) supplemented with 10% heat-inactivated human pooled AB serum, 2 mmol/L L-glutamine, 20 nmol/L HEPES, and 100 units/mL penicillin/streptomycin. PBMC were labeled with CFSE (Molecular Probes) and cultured at 37°C, in 5% CO_2, at 10^6/mL in 96-well flat-bottomed plates (0.2 mL/well), with isopentenyl PPi (IPP, Sigma Chemical Co.; 10^{-5} mol/L final concentration) and 20 units/mL IL-2 (23, 25). Seven days later, cells were collected and the percentage of Vγ9Vδ2 cells within the $CD3^+$ population was assessed by fluorescence-activated cell sorting (FACS). 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 in the absence of IPP (23). IFNγ levels in the 48 h culture supernatants were assessed by two mAbs sandwich ELISA (23) following manufacturer's recommendations (R&D Systems). Nα-CBZ-L-lysine-thiobenzyl (BLT)-esterase levels in 24 h culture supernatants were determined by the BLT esterase assay. Briefly, 20 μL culture supernatant were incubated with 35 μL 1 mmol/L BLT (Sigma), 35 μL 1 mmol/L 5-5′-dithio-bis-(2-nitrobenzoic acid; Sigma), and 10 μL 0.1% Triton X-100 (Sigma). After incubation for 30 min at 37°C, absorbance was measured at 405 nm (23).

Intracellular staining for IFN-γ and perforin. PBMC stimulated with IPP as described above, in the presence of monensin for 6 h at 37°C in 5% CO_2 were harvested, washed, and stained with anti-Vδ2 mAb in incubation buffer (PBS containing 1% FCS and 0.1% Na azide) for 30 min at 4°C. Cells were then washed twice in PBS with 1% FCS and fixed with PBS containing 4% paraformaldehyde overnight at 4°C. Fixation was followed by permeabilization with PBS containing 1% FCS, 0.3% saponin, and 0.1% Na azide for 15 min at 4°C, and fixed permeabilized cells were stained with an anti–IFN-γ antibody. After two more washes in PBS containing 1% FCS, the cells were analyzed by FACSCalibur. Lymphocytes were gated by forward and side scatter and analysis done on 100,000 acquired events for each sample. For detection of intracellular perforin, PBMC were stained directly

ex vivo (i.e., without any antigen stimulation *in vitro*) in calcium-free medium with anti-Vδ2 mAb as above described, washed, and fixed with PBS containing 4% paraformaldehyde for 30 min at 4°C. After two washes in permeabilization buffer, cells were stained with antiperforin antibody (δG2, Alexis; 2 μg/mL); after two more washes in PBS + 1% FCS, the cells were analyzed as above.

Analysis of tumor necrosis factor–related apoptosis inducing ligand expression. PBMC from healthy volunteers were stimulated *in vitro* for 3 days with zoledronate at the indicated concentrations, with or without IL-2 (100 units/mL). Brefeldin A (Sigma) was added to the cultures at 10 μg/mL 3 h before harvest, and surface-stained cells (CD3-ECD and Vγ9-PE-Cy5, Beckman Coulter) were then labeled using the Fix & Perm kit (Caltag) with anti-TRAIL (tumor necrosis factor–related apoptosis inducing ligand)-phycoerythrin (BD Biosciences). TRAIL in sera was detected using a human TRAIL DuoSet ELISA kit (R&D Systems).

Statistical analysis. Kaplan-Meier curves were used to assess the effect of treatments on 12-month survival; a sample size calculation of 18 patients (nine per group) assured a power of 85% in detecting a 3-month difference in survival curves among groups, with a SD of ±3 and a two-sided α error of 5. The significance of the differences in survival was assessed using the log-rank, Breslow (Generalized Wilcoxon), or Tarone-Ware tests. Data from more than two groups were compared using one-way ANOVA with Tukey-Kramer multiple comparison test using Instat software (version 3.05, GraphPad). *P* values <0.05 were considered statistically significant.

Results

Rationale. Human Vγ9Vδ2 T cells can be activated in a non-MHC–dependent manner by phosphoantigens or by agents that provoke the accumulation of endogenous PPi such as IPP. Among such agents, aminobisphosphonates are already widely used in the clinic, for example, to diminish osteoclast activity in osteoporosis or in elderly patients receiving chronic steroid treatment. By several criteria, zoledronate is the most potent and efficacious clinically approved aminobisphosphonate (29, 30). Our ongoing molecular analyses of Vγ9Vδ2 T-cell responses have indicated that IL-2 up-regulates several effector molecules that may be efficacious in tumor therapy, and is active *in vivo* on Vγ9Vδ2 T cells at a lower concentration than that required to affect αβ T cells or natural killer (NK) cells. For these reasons, we rationalized that zoledronate ± low-dose IL-2 may provide a feasible and safe means to activate Vγ9Vδ2 T cells *in vivo*.

Toxicity. One to 3 days after the first administration, six of nine patients treated with zoledronate + IL-2 (67%) and two of nine patients treated with zoledronate alone (22%) developed a transient flu-like syndrome that was easily controlled by oral paracetamol. This side effects was expected as it was also detected after application of pamidronate and IL-2 to multiple myeloma and non–Hodgkin's lymphoma patients (24). Two patients (22%) receiving zoledronate + IL-2 developed a local erythema at the site of IL-2 administration. No other hematologic, hepatic, renal, or neurologic toxicity, or allergic, autoimmune, or fatigue side effect was observed during the treatment.

γδ T-cell responses. Phenotypic and functional responses of γδ T cells were measured before and at 3, 9, and 12 months after beginning treatment with zoledronate ± IL-2 (Fig. 1*A* and *B*). By multiple criteria, the surviving patients in the two treatment protocols showed substantially different effects on γδ cells. Before treatment, almost all patients displayed $\sim 25 \times 10^3$ to 45×10^3 γδ cells per milliliter of blood. As four patients treated with zoledronate alone (cohort A) died before month 3, longitudinal analysis was possible in only five such patients (1A, 2A, 3A, 7A, and 8A). Of these, only patient 7A sustained starting levels of γδ cells across the treatment period. Although patient 1A showed a transient increase in γδ cell numbers at 3 months, the cells then declined to below starting levels.

In stark contrast, seven patients treated with zoledronate + IL-2 (cohort B) survived past month 12, of whom five (1B, 2B, 3B, 4B, and 8B) showed sustained or increased numbers of peripheral γδ T cells across the full period. One surviving patient (9B) showed a decrease in peripheral γδ T cells, up to 9 months, although those numbers then stabilized, whereas another (6B) showed a more substantial ongoing decline. Both of the two cohort B patients who died (5B and 7B) displayed precipitous and progressive decreases in peripheral blood γδ T-cell numbers. In addition to the different frequencies of peripheral blood γδ cells in the two cohorts, there was a more pronounced increase in CD69 (Fig. 1*C*) and/or HLA-DR (Fig. 1*D*) antigens on γδ T cells in cohort B, indicating greater activation *in vivo*. The stimulatory effects of zoledronate ± IL-2 treatment were largely restricted to γδ T cells, because neither absolute numbers nor CD69 and HLA-DR expression were altered on αβ T cells or NK cells (Supplementary Table S2).

When a subset analysis was done on γδ T cells in the seven cohort B patients surviving through month 12, there were in all cases sharp and progressive decreases in absolute numbers of cells with naïve-like and central memory–like phenotypes (T_{naive} and T_{CM} γδ T cells), and, correspondingly, albeit more variable increases in cells with effector memory–like and terminally differentiated phenotypes (T_{EM} and T_{EMRA} γδ cells), although the T_{EMRA} γδ cells declined somewhat by 12 months (Fig. 2). The pattern of increased numbers and an altered phenotype of peripheral γδ cells was strikingly depicted by the raw data for

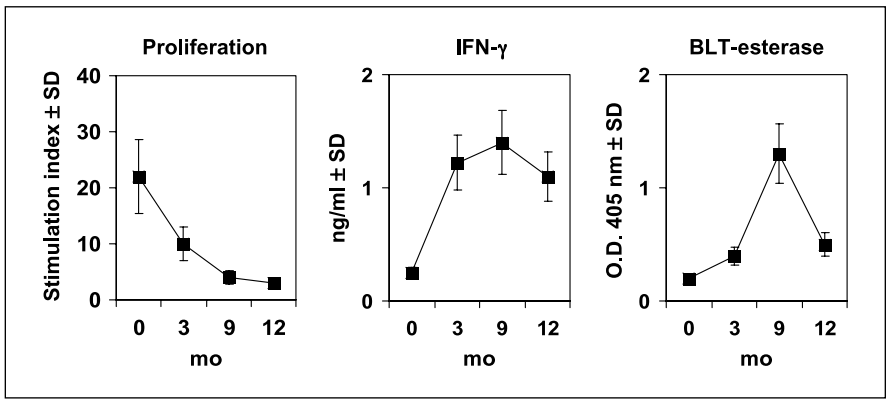

Figure 3. Proliferation, IFN-γ production, and cytotoxic activity of γδ T cells in patients treated with zoledronate + IL-2. PBMC were obtained from seven patients treated with zoledronate + IL-2, before starting treatment (month 0), and 3, 9, and 12 mo after treatment. Cells were cultured *in vitro* with IPP and IL-2, as described in Materials and Methods. Proliferation was evaluated 7 d later, whereas IFN-γ production and BLT-esterase activities were assessed in the 48 and 24 h supernatants, respectively. *O.D.,* absorbance. *Points,* mean; *bars,* SD.

patient 1B shown in Supplementary Fig. S1. Overall, the statistically significant changes indicate that zoledronate + IL-2 provokes a long-term effector maturation and mobilization of peripheral blood γδ T cells and evoke our previous observations after zoledronate injection in cancer patients (25). Indeed, a similar trend was seen in the three patients treated with zoledronate alone surviving more than 12 months (data not shown), although the absolute numbers of peripheral γδ T cells declined in all but one of these patients (7A).

The phenotypic modifications of γδ T cells in cohort B were paralleled by modifications in functional responses *in vitro*. As shown in Fig. 3, proliferative responses to IPP declined over time, whereas the capacity to produce IFN-γ and to release BLT-esterase (as a variable of cytotoxicity) consistently increased, peaking at 9 months. Again, this trend is illustrated by raw data for patient 1B (Supplementary Fig. S2).

Clinical responses and clinical correlates. Only two of nine patients treated with zoledronate alone showed any clinical response (Supplementary Table S1, Fig. 1*A*). In one patient (1A), stable disease was observed over 14 months, whereas patient 7A, who was the only patient in cohort A to sustain pretreatment levels of γδ T cells, achieved a partial remission. In contrast, six of nine patients treated with zoledronate + IL-2 showed favorable clinical responses (Supplementary Table S1; Fig. 1*B*). Two patients (1B and 8B) achieved a partial remission, and each showed aggregate increases in γδ T-cell numbers across the treatment period. Four additional patients (2B, 3B, 4B, and 9B) achieved stable disease, lasting 14 to 16 months, and with a sole exception (9B) each showed sustained γδ T-cell numbers; indeed, even in 9B, γδ T-cell numbers had stabilized by 9 to 12 months at $\sim 10 \times 10^3$ γδ T cells per milliliter of blood. By contrast, the deterioration of patients 5B, 6B, and 7B was in each case preceded by a substantial decline in γδ T-cell numbers (Fig. 1*A* and *B*). The 12-month survival curves of patients treated with zoledronate alone and those treated with zoledronate + IL-2 is shown in Fig. 4*A*. Figure 4*B* shows echo-color Doppler imaging of the prostate of patient 1B before (*top*) and 12 months (*bottom*) after zoledronate + IL-2 therapy, showing regression of prostate malignancy.

The prima facie correlation between 12-month clinical outcome (i.e., partial remission versus stable disease versus progression) and, respectively, increases in, maintenance of, or decreases in circulating γδ T-cell numbers was provocative and was investigated further. A statistically significant correlation existed between favorable outcome at 12 months, and either total γδ cell numbers or the numbers of TCR γδ T_{EM} cells measured at 9 months (Fig. 5*A* and *B*). Moreover, a statistically significant inverse correlation was also found between either total γδ T cells or TCR γδ T_{EM} cells and serum PSA levels at 9 months (Fig. 5*C* and *D*). Of note, we did not find any correlation between pretreatment PSA levels and total γδ cells ($r = 0.0033$, $P = 0.99$) or their T_{EM} subset ($r = 0.088$, $P = 0.81$). To illustrate this point further, Supplementary Fig. S3*A* and *B* shows a longitudinal analysis of total γδ cells, the γδ T_{EM} subset, and serum PSA levels in a patient (1B) achieving an objective immune response (partial remission), and in a patient (6B) showing disease progression.

The highly distinct clinical outcomes in the two cohorts prompted us to examine further the differences that might exist between them. Related to this, a set of microarray analyses was undertaken while the trial was ongoing, of human peripheral blood γδ cells stimulated with a phosphoantigen TCR agonist (HMB-PP) in the presence of different cytokines, including IL-2 (31). The study confirmed that IFN-γ and tumor necrosis factor-α are up-regulated in such circumstances, but additionally found that the cytolytic mediator, TRAIL, was specifically up-regulated in the presence of IL-2. We therefore tested whether TRAIL was likewise up-regulated *in vitro* by zoledronate + IL-2, more than by zoledronate alone, and confirmed that it was, identifying a provocative potential difference between the two cohorts (Fig. 6*A*). Although these findings postdated the analysis of patients' γδ T cells, an a posteriori examination of patients' serum showed that TRAIL levels were essentially maintained in cohort B, whereas they mostly declined in cohort A (Fig. 6*B*). Of note, the single outlier in cohort A (7A) whose TRAIL levels had recovered close to their pretreatment

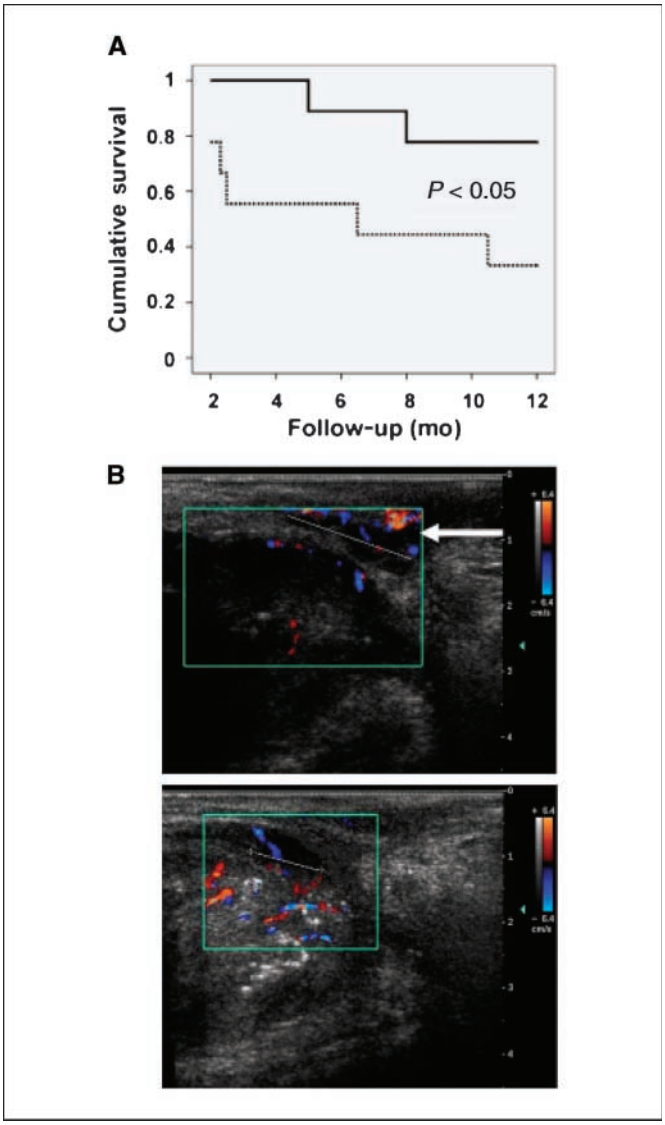

Figure 4. *A,* Kaplan-Meier curves for 12-mo survival according to treatment with zoledronate (*dotted lines*) or zoledronate + IL-2 (*black-filled lines*). $P < 0.05$ by the log-rank, Breslow (generalized Wilcoxon) or Tarone-Ware tests. *B,* echo-color Doppler imaging of the prostate of patient 1B before (*top*) and 12 mo (*bottom*) after zoledronate + IL-2 therapy. *White arrow (top),* an area of hypervascularization at the level of a hypoechogenic nodule (*the darkest area surrounding the white dotted line*), which contains malignancy. Twelve months after therapy, the hypoechogenic area is reduced and only one normal vessel is found in its context, indicating tumor regression. *Green box,* the area in which the echo-color Doppler signal was registered.

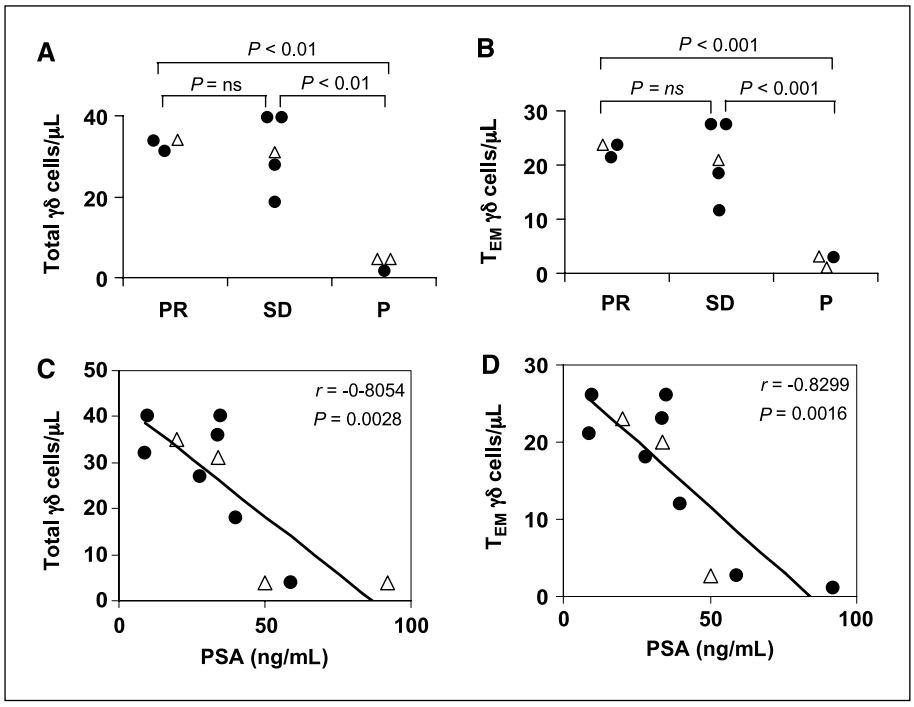

Figure 5. Correlation between $\gamma\delta$ T-cell numbers and clinical outcome. *A,* numbers of total $\gamma\delta$ cells and (*B*) V$\delta2^+$ $\gamma\delta$ T_{EM} cells assessed at 9 mo after therapy are shown in three patients with partial remission, five patients with stable disease, and three patients with progression. ●, patients treated with zoledronate + IL-2; △, patients treated with zoledronate alone. *C,* inverse correlation between numbers of total $\gamma\delta$ cells and (*D*) of V$\delta2^+$ $\gamma\delta$ T_{EM} cells and PSA levels, as assessed at 9 mo after therapy, in three patients with partial remission, five patients with stable disease, and three patients with progression (*P*). ●, patients treated with zoledronate+IL-2; △, patients treated with zoledronate alone.

levels by 9 months was also the one patient treated with zoledronate alone who maintained $\gamma\delta$ T-cell numbers and who showed a partial remission. When serum TRAIL levels at 9 months were examined for all 11 patients surviving at 12 months, there was a clear trend whereby higher TRAIL levels were associated with improved clinical outcome (Fig. 6*C*); in fact, there was only a single overt outlier (6B) who was treated with zoledronate + IL-2 and who maintained TRAIL levels, but whose disease progressed. This patient prevented the correlation between TRAIL levels and outcome reaching statistical significance. Nonetheless, the likely

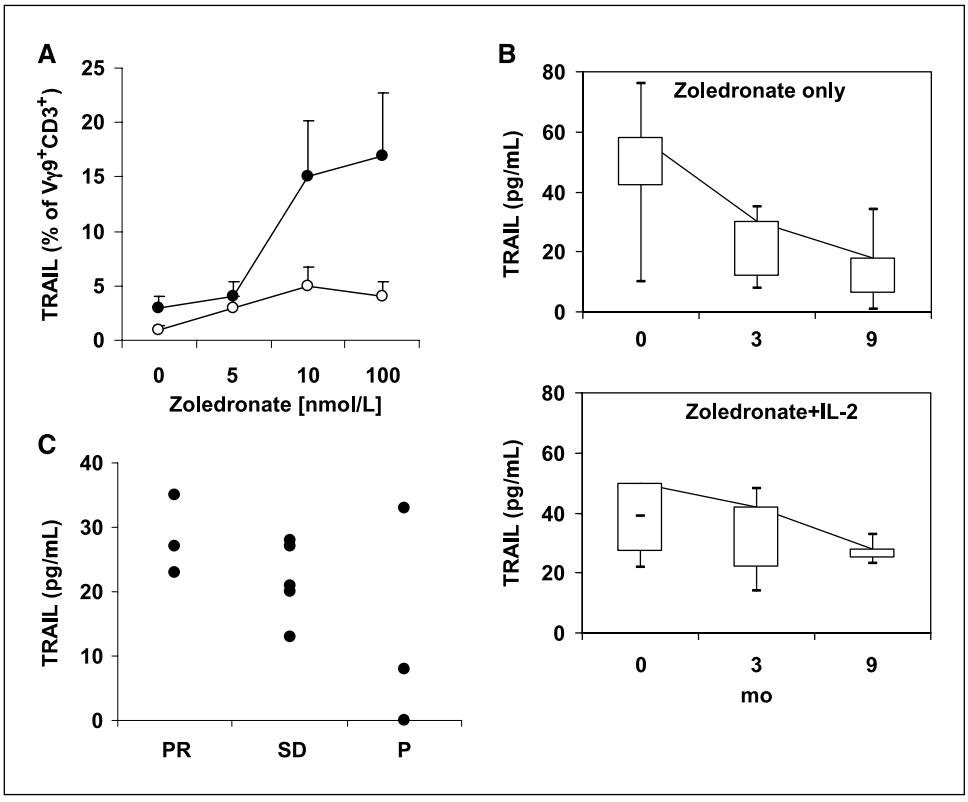

Figure 6. Flow cytometry for intracellular TRAIL on cultured PBMC (*A*), influence of treatment (zoledronate ± IL-2) on serum TRAIL concentrations (*B*), and association between TRAIL concentrations and clinical outcome (*C*). *A,* percentage of V$\gamma9^+$CD3^+ cells positive for TRAIL on day 3 (n = 5 healthy volunteers). *Points,* mean; *bars,* SE. *B,* TRAIL concentration in serum of patients treated with zoledronate only or zoledronate + IL-2, analyzed at indicated time points. See the legend to Fig. 2. *C,* TRAIL concentrations assessed at 9 mo after therapy are shown for three patients with partial remission, five patients with stable disease, and three patients with progression. *Lines,* median values.

biological significance of the correlation between clinical outcome and serum TRAIL concentrations at 9 months was highlighted by the lack of an obvious correlation with other variables, including pretreatment TRAIL levels (Supplementary Fig. S4), and 9-month levels of other immunologic mediators (data not shown).

Discussion

Several observations in mice and in humans have collectively laid the foundation for examining the potential of γδ T cells to exert tumor immunotherapy. A practical issue has been how to accomplish this. In this regard, an attractive feature of human peripheral blood Vγ9Vδ2 T cells is their capacity to be readily and specifically activated either by low molecular mass phosphoantigens, such as HMB-PP or IPP, or by agents that provoke IPP accumulation. Among the latter, aminobisphosphonates are well established in the clinic, and extensive data are available on the antiangiogenic, antiosteolytic, and proapoptotic properties of the compounds (29, 30).

In patients with multiple myeloma or with low-grade non–Hodgkin lymphoma, occurrences of acute-phase reaction to i.v. pamidronate were attributed to the systemic activation of phosphoantigen-reactive γδ T cells (24), and this provoked the deliberate treatment of lymphoma patients with pamidronate and low-dose IL-2. This achieved some promising results after patients were prescreened for those whose γδ T cells would substantively respond to pamidronate + IL-2 *in vitro* (24). By several criteria, zoledronate is more potent and efficacious than pamidronate, and previous studies by our own group in patients with breast (n = 3) and prostate (n = 6) tumors showed that zoledronate induced *in vivo* the maturation of peripheral blood γδ T cells into more potent cytotoxic and IFN-γ–secreting cells (25). This notwithstanding, there has hitherto been no study of the consequences of a selective activation of γδ T cells *in vivo* in patients with late-stage, metastatic carcinomas, a significant unmet clinical need. Moreover, there has been no assessment of the consequences of administering zoledronate *in vivo* in the presence or absence of a specific cytokine, such as IL-2. This is important given the evidence that the differentiation of γδ cells, like other T cells, is substantially influenced by the prevailing cytokine milieu (31–34). Given such paucity of existing data, the assessment of toxicity was a major aim of the current study. Our findings reveal that the combination of zoledronate + IL-2 was well tolerated, with no long-term overt toxicity observed. Consistent with a lack of significant side effects, neither zoledronate nor IL-2 used at the low doses described in this study had any measurable effects on either αβ T cells or NK cells (Supplementary Table S2).

A second aim of our study was to assess whether reproducible phenotypic changes were induced in the γδ T-cell compartment. In this regard, the most striking effect was the long-term and substantial differentiation of γδ T cells toward an effector-memory–like phenotype, with absolute numbers of such cells particularly enhanced in patients treated with zoledronate + IL-2; indeed, absolute numbers of peripheral T_{naive} and T_{CM} γδ cells decreased by 3 months after treatment and had virtually disappeared at 9 to 12 months. This was seen in the majority of zoledronate + IL-2–treated patients without any prescreening for those whose cells would respond to this regimen *in vitro*. Based on variable γδ T-cell repertoires and on the preexisting status of such cells, it is known that peripheral blood responses to phosphoantigens or aminobisphosphonates show individual variation, and by analogy to earlier studies in lymphoma (24), one can only assume that such prior screening may well improve the response rate to zoledronate + IL-2. A similar trend was seen in three of nine patients receiving zoledronate alone, although they were, with one exception (7A), less impressive, and may have been sustained by individually variable levels of endogenous cytokines.

A third and final aim of our study was to assess whether there was a differential clinical response to the treatment regimens. Indeed, whereas only two of nine patients treated with zoledronate achieved an objective tumor response, this was true for 67% of those treated with zoledronate + IL-2, where the response at 12 months showed a correlation with the absolute numbers of total γδ T cells; with the numbers of γδ T_{EM} cells; and although not significantly, with serum levels of TRAIL measured at 9 months posttreatment. Moreover, the fact that clinical outcome was influenced by the type of regimen used to activate γδ T cells strongly suggests that γδ T-cell activation is at least partially causal to the response. Thus, γδ T cells stimulated *in vitro* with zoledronate and IL-2 possess an increased capacity for killing tumor cells *in vitro* (30),[6] whereas our immunologic monitoring provided no evidence that zoledronate ± low-dose IL-2 induced activation, expansion, and/or differentiation of either αβ T cells or NK cells (note that the concentration of IL-2 used here is much lower than that used in other immunotherapies; refs. 35–37). Interestingly, it was recently reported that the presence of T_{EM} cells within colorectal cancer correlates with the absence of pathologic evidence of early metastases and with prolonged survival (38). However, despite the provided *in vitro* evidence that stimulation with zoledronate + IL-2 increases the production of TRAIL by γδ cells in short-term cultures, we have actually no proof that γδ cells solely contribute to sustained serum TRAIL levels in patients treated with zoledronate + IL-2. The finding that neither absolute numbers nor CD69 and HLA-DR expression were altered on αβ T cells or NK cells (Supplementary Table 2) strongly points to a selective effects on γδ cells. The fact that patients enrolled in this study were at a terminal stage of disease might also contribute to the lack of increase in serum TRAIL levels.

We have yet to elucidate the key contributions to tumor regulation made by γδ T_{EM} cells induced by zoledronate + IL-2. Nonetheless, clues should exist in the ongoing microarray analysis of Vγ9Vδ2 cells stimulated via the TCR in the presence of IL-2. This study has already provoked our analysis of TRAIL as a potential active component, and, guided by this, future studies will look directly at TRAIL production by γδ T cells in an immunotherapy context. For several reasons, TRAIL is an attractive candidate: For example, it kills many tumor cell lines but not most nontransformed cells, and the selective efficacy of histone deacetylase inhibitors versus acute myeloid leukemia cells involves TRAIL induction *in vivo* (39, 40). Nonetheless, there is a view that the results using agonistic anti–TRAIL receptor antibody as an antitumor agent have been disappointing, perhaps because of resistance to TRAIL-mediated apoptosis, which is common in cancer cells (41). Interestingly, resistance can be overcome by IFN-γ (42), which is produced in high amounts by zoledronate + IL-2–stimulated Vγ9Vδ2 T cells, and which is positively reinforced by signaling via TRAIL, and vice versa (43, 44). Thus, it is the maintenance of TRAIL production in the context of other products

[6] Our unpublished results.

of Vγ9Vδ2 T_{EM} cells that may be key, consistent with which clinical outcome did not correlate with pretreatment levels of TRAIL. This emphasizes a major potential advantage of cell-based therapy. Indeed, the combination of an agonistic anti–TRAIL receptor antibody and the induction of IFN-γ–producing T cells was shown to eradicate established tumors in mice (45).

The encouraging prospect that the activation of peripheral blood Vγ9Vδ2 T cells can be efficacious against solid tumors requires further follow-up, including an analysis of tumor-infiltrating lymphocytes to assess whether the activated cells are indeed infiltrating the tumors and/or are helping other cells to do so. Wang and colleagues recently reported that human prostate and breast carcinoma are naturally and strongly infiltrated by TRAIL-producing γδ T cells,[7] suggesting the value of analyzing the regulation on zoledronate + IL-2–stimulated-Vγ9Vδ2 T cells of trafficking molecules and of molecules such as NKG2D that may contribute to tumor recognition (46, 47). The improved cell surface definition of an antitumor Vγ9Vδ2 T cell, coupled with the correlation of clinical outcome with the numbers of T_{EM} γδ T cells, also suggests that in the short-term, the peripheral blood analysis of the status of γδ cells, and of TRAIL, may provide a useful biomarker of a patient's antitumor response upon therapy with zoledronate + IL-2.

[7] γδ T Cell Conference; 2006 Mar 26–28; La Jolla, CA.

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