

marrow complete remission (CR), and standard non-Hodgkin lymphoma (NHL) doses of rituximab in untreated CLL patients<sup>4</sup> cleared the bone marrow in only 9% of patients. We, therefore, would offer an alternative focus for future investigation with rituximab. Specifically, efforts should be focused on understanding potential factors such as stromal cell interaction that disrupt apoptosis,<sup>5</sup> CLL cell density, antibody penetration, and decreased access to complement and effector cells within the bone marrow that might prevent efficient clearance of tumor cells in the majority of patients. Such studies will facilitate development of alternative combination strategies to improve bone marrow responses with this promising combination therapy.

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## To the editor:

### Induction of $\gamma\delta$ T-lymphocyte effector functions by bisphosphonate zoledronic acid in cancer patients in vivo

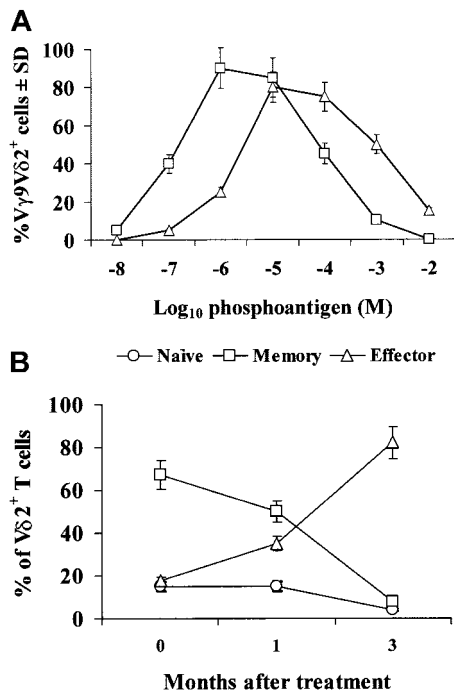
Bisphosphonates are analogues of endogenous pyrophosphate in which a carbon atom replaces the central atom of oxygen. This carbon substitution makes these compounds resistant to hydrolysis and allows 2 additional chains of variable structure. One of these side chains usually contains a hydroxyl moiety that allows high affinity for calcium crystals and bone mineral. The differences at the other side chain produce marked differences in the antiresorptive potency of different bisphosphonates. In fact, the newer bisphosphonates, such as ibandronate and zoledronic acid, show 10 000- to 100 000-fold more potency than the older agents such as etidronate.<sup>1</sup>

Exposure to bisphosphonates may not only reduce bone loss but may also have an antimyeloma effect. Recent animal studies have shown that the aminobisphosphonates also have potent antiangiogenic activity that may contribute to these drugs' antitumor effect as well as provide additional mechanisms by which these drugs may have antimyeloma effects as well.<sup>1</sup>

A new potential antitumor mechanism for these compounds was recently reported for aminobisphosphonates.<sup>2</sup> These drugs were shown to induce expansion of  $V\gamma 9V\delta 2$  T cells in peripheral blood mononuclear cell cultures and enhance cytotoxicity of malignant plasma cells in bone marrow cultures by these  $V\gamma 9V\delta 2$  T lymphocytes. Thus, there is increasing evidence that bisphosphonates, especially the nitrogen-containing compounds, can lead to direct and indirect effects that result not only in less bone loss but less tumor burden as well. In support of this, Wilhelm et al<sup>3</sup> now report the results of a pilot study of low-dose interleukin-2 (IL-2) in combination with pamidronate in patients with low-grade non-Hodgkin lymphoma or multiple myeloma. They selected 9 patients by positive in vitro proliferation of  $V\gamma 9V\delta 2$  T cells in response to pamidronate and IL-2, and show that 5 of the patients had significant in vivo activation/proliferation of  $V\gamma 9V\delta 2$  T cells. Three patients also achieved objective responses, indicating that  $V\gamma 9V\delta 2$  T cells might contribute to this antilymphoma effect.

To evaluate the antitumor activity of  $V\gamma 9V\delta 2$  T cells in vivo, we initiated a pilot study of low-dose zoledronic acid in 9 cancer patients with bone metastases (3 females affected by breast cancer; 6 males affected by prostate cancer; median age, 66 years [range, 54-83 years]). The objective of this study was to evaluate the in vivo effect of zoledronic acid on recently identified subsets of  $V\gamma 9V\delta 2$  cells that display different functional activities:  $CD45RA^+CD27^+$  naive and  $CD45RA^-CD27^+$  memory  $V\gamma 9V\delta 2$  cells strongly proliferate but lack immediate effector functions, while  $CD45RA^-CD27^-$   $V\gamma 9V\delta 2$  cells proliferate poorly but produce interferon  $\gamma$  (IFN $\gamma$ ) and exert cytotoxicity.<sup>4</sup> Both of these functions are strictly involved in their antitumor activity. Patients were treated with 4 mg zoledronic acid via 15-minute intravenous infusion every 3 weeks. Peripheral blood mononuclear cells (PBMCs) were collected before treatment (time 0), and 1 month (time 1) and 3 months (time 2) after the first administration. PBMCs were also taken from 15 age- and sex-matched healthy control subjects.

Preliminary experiments (Figure 1A) showed that zoledronic acid was as able as the phosphoantigen isopentenylpyrophosphate (IPP) to induce expansion of  $V\gamma 9V\delta 2^+$  T cells upon culture in vitro in the presence of IL-2. The relative percentages of naive, memory, and effector subsets of  $V\gamma 9V\delta 2$  cells were comparable in control and cancer patients before treatment with zoledronic acid (data not shown). However, treatment with zoledronic acid caused a decrease in the percentages of naive and memory  $V\gamma 9V\delta 2$  cells, which was evident 1 month after the initial treatment and dramatic after 3 months (Figure 1B): in fact, at this stage, naive and memory cell subsets were less than 10% of the whole  $V\gamma 9V\delta 2$  population, while in control subjects these 2 subsets usually account for about 70% of  $V\gamma 9V\delta 2$  cells. The decrease of naive and  $V\gamma 9V\delta 2$  cells was paralleled by an increase in the percentage of the effector subset, which accounted for about 80% of the whole  $V\gamma 9V\delta 2$  population after 3 months from the initial treatment with zoledronic acid. As the

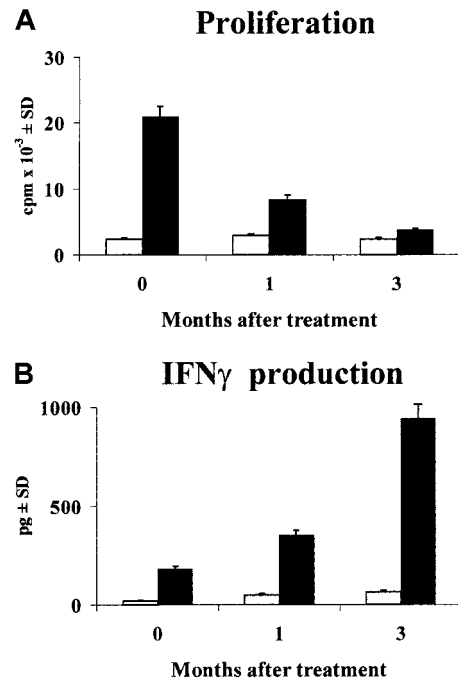


**Figure 1. Zoledronic acid activates in vitro V $\gamma$ 9V $\delta$ 2 cells and induces subset distribution in cancer patients in vivo.** (A) PBMCs were incubated in vitro with IPP ( $\Delta$ ) or zoledronic acid ( $\square$ ) at the indicated final concentrations and 10 U/mL IL-2. Then, 7 days later, cells were collected and the percentage of V $\gamma$ 9V $\delta$ 2<sup>+</sup> cells within the CD3<sup>+</sup> population was assessed. (B) V $\gamma$ 9V $\delta$ 2 T-cell subsets were analyzed by 3-color fluorescence-activated cell-sorter (FACS) analysis before or after in vivo treatment with zoledronic acid. The following antibodies were used in different combinations: anti-V $\delta$ 2 fluorescein isothiocyanate (FITC, IMMU389; Coulter, Miami, FL), anti-CD27 phycoerythrin (PE) (M-T271; BD Pharmingen, San Diego, CA), and anti-CD45RA PE–cytochrome 5 (Cy5, 2H4; Coulter). Data were acquired on a FACSCalibur instrument (BD Biosciences, San Diego, CA) and analyzed using CellQuest software (BD Immunocytometry Systems, San Jose, CA). Error bars indicate standard deviation.

percentage and absolute numbers of V $\gamma$ 9V $\delta$ 2 cells in PBMCs were not modified during treatment, we interpret these results to indicate that zoledronic acid in vivo expands a subset of effector V $\gamma$ 9V $\delta$ 2 cells, while decreasing the naive and memory subsets.

Different subsets of V $\gamma$ 9V $\delta$ 2 cells have been reported to exert different functions.<sup>4</sup> Therefore, we assessed whether modification of V $\gamma$ 9V $\delta$ 2 cell subset distribution was accompanied by alterations in the functions they usually perform. As shown in Figure 2A, proliferative activity of V $\gamma$ 9V $\delta$ 2 cells upon in vitro stimulation with IPP, which is a property of naive and memory cells, strongly decreased upon in vivo treatment with zoledronic acid. Conversely, production of IFN $\gamma$  by V $\gamma$ 9V $\delta$ 2 cells upon in vitro stimulation with IPP, which is a property of effector cells, consistently increased upon in vivo treatment with zoledronic acid.

Although the data here reported strengthen the results of Wilhelm et al<sup>3</sup> (ie, the high IFN $\gamma$  concentrations in serum and the slow response profiles of most of lymphoma patients), a major difference to the study by Wilhelm et al is that they used a combination of pamidronate and low-dose IL-2 in vivo. In principle, IL-2 application in vivo might not have influenced their results, as indicated by the findings that aminobisphosphonate-induced V $\gamma$ 9V $\delta$ 2 T-cell activation and IFN $\gamma$  production is strictly dependent on the presence of monocytes,<sup>5</sup> and that V $\gamma$ 9V $\delta$ 2 T cells can be quickly and antigen-nonspecifically activated, even in the absence of IL-2, by monocyte-derived IL-12 and tumor necrosis factor  $\alpha$  (TNF $\alpha$ ).<sup>6</sup>



**Figure 2. Proliferation and IFN $\gamma$  production by V $\gamma$ 9V $\delta$ 2 cells from cancer patients before and after treatment with zoledronic acid.** PBMCs were cultured at 37°C, in the presence of 5% CO<sub>2</sub>, at 10<sup>6</sup>/mL in 96-well flat-bottomed plates (0.2 mL/well), with isopentenylpyrophosphate (IPP; Sigma Chemical, St Louis, MO; 100  $\mu$ M/mL final concentration) and 20 U/mL final concentration human recombinant IL-2 ( $\blacksquare$ ) or with IL-2 alone as a control ( $\square$ ). Proliferation (A) was measured 7 days later by adding 1  $\mu$ Ci/well (0.037 MBq) [<sup>3</sup>H]thymidine (Amersham, Arlington Heights, IL) during the last 6 hours of culture. Cells were then harvested and [<sup>3</sup>H]thymidine incorporation was measured with a liquid scintillation  $\beta$ -counter. Results are expressed as mean counts per minute (cpm) of triplicate wells  $\pm$  standard deviation (SD). IFN $\gamma$  levels in the 48-hour culture supernatants (B) were assessed by 2 monoclonal antibodies (mAbs) sandwich enzyme-linked immunosorbent assay (ELISA) assay following the manufacturer's recommendations (R&D Systems, Minneapolis, MN).

All together, our results indicate that in vivo treatment with zoledronic acid induces V $\gamma$ 9V $\delta$ 2 cells to mature toward an IFN $\gamma$ -producing effector phenotype, which may induce more effective antitumor responses.

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