High Phosphoantigen Levels in Bisphosphonate-Treated Human Breast Tumors Promote Vγ9Vδ2 T-Cell Chemotaxis and Cytotoxicity In Vivo

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ABSTRACT

The nitrogen-containing bisphosphonate zoledronic acid (ZOL), a potent inhibitor of farnesyl pyrophosphate synthase, blocks the mevalonate pathway, leading to intracellular accumulation of IPP/Apppl mevalonate metabolites. IPP/Apppl accumulation in ZOL-treated cancer cells may be recognized by Vγ9Vδ2 T cells as tumor phosphoantigens in vitro. However, the significance of these findings in vivo remains largely unknown. In this study we investigated the correlation between the anticancer activities of Vγ9Vδ2 T cells and the intracellular IPP/Apppl levels in ZOL-treated breast cancer cells in vitro and in vivo. We found marked differences in IPP/Apppl production among different human breast cancer cell lines post-ZOL treatment. Coculture with purified human Vγ9Vδ2 T cells led to IPP/Apppl-dependent near-complete killing of ZOL-treated breast cancer cells. In ZOL-treated mice bearing subcutaneous breast cancer xenografts, Vγ9Vδ2 T cells infiltrated and inhibited growth of tumors that produced high IPP/Apppl levels, but not those expressing low IPP/Apppl levels. Moreover, IPP/Apppl not only accumulated in cancer cells but it was also secreted, promoting Vγ9Vδ2 T-cell chemotaxis to the tumor. Without Vγ9Vδ2 T-cell expansion, ZOL did not inhibit tumor growth. These findings suggest that cancers producing high IPP/Apppl levels after ZOL treatment are most likely to benefit from Vγ9Vδ2 T-cell–mediated immunotherapy.

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INTRODUCTION

Nitrogen-containing bisphosphonates (NBPs) are potent inhibitors of osteoclast-mediated bone resorption and can treat bone-loss disorders such as postmenopausal osteoporosis (1), aromatase inhibitor-associated bone loss, and cancer treatment-induced bone loss (2). In preclinical models, NBPs have direct and indirect antitumor properties (3). NBPs inhibit farnesyl pyrophosphate synthase (FPPS), thus blocking the mevalonate pathway and preventing prenylation of G-proteins (e.g., Ras and Rho). In addition to effects on bone, this can inhibit tumor-cell invasion, proliferation, and adhesion (3). Additional studies suggest that NBPs may also inhibit angiogenesis (3). Importantly, there is now clinical evidence that adding the NBP zoledronic acid (ZOL) to endocrine therapy improves disease-free survival in estrogen-responsive early breast cancer (4,5) and that adding ZOL to neoadjuvant chemotherapy reduces residual invasive breast cancer tumor size (6). How ZOL mediates this activity is not understood.

Emerging data suggest that NBPs have immunomodulatory properties (7,8), which may have therapeutic applications. Some NBPs can induce expansion of human Vγ9Vδ2 T cells, the dominant subset of γδ T cells (8-10). NBP internalization by peripheral blood mononuclear cells (PBMCs) inhibits FPPS, leading to intracellular accumulation of isopentenyl pyrophosphate (IPP) that, in turn, stimulates Vγ9Vδ2 T-cell proliferation in a Vγ9Vδ2 T-cell receptor (TCR)-dependent manner (11,12). The ATP analogue Apppi (triphosphoric acid l-adenosin-5'-yl ester 3-[3-methylbut-3-enyl] ester), which results from covalent binding of IPP and AMP (13), can also act as a phosphoantigen, stimulating the expansion of Vγ9Vδ2 T cells from PBMCs (14).

In preclinical models, Vγ9Vδ2 T cells activated by NBPs have potent antitumor effects in vitro (7, 15-18), and Vγ9Vδ2 T cells expanded in vitro maintain antitumor activity upon adoptive transfer into immunodeficient mice inoculated with melanoma or pancreatic adenocarcinoma cells (15). ZOL also enhances Vγ9Vδ2 T-cell cytotoxicity in experimental models of chronic myelogenous leukemia, lung, colon or bladder cancer (18-21).
Additionally, clinical evidence suggests anticancer effects of the Vγ9Vδ2 T cells in patients with lymphoid malignancies treated with the NBP pamidronate + interleukin-2 (IL-2) (7, 22), and in castration-resistant prostate cancer patients and advanced metastatic breast cancer patients treated with ZOL + IL-2 (23,24). A single dose of ZOL has also been shown to durably activate γδ T cells in patients with breast cancer (25).

Cancer cells may be especially vulnerable to Vγ9Vδ2 T cells (10, 11), even though the antitumor activity of Vγ9Vδ2 T cells against different tumor cells treated with NBPs varies (26). Interestingly, IPP/ApppI production after NBP treatment in different cell lines also varies (27) and these mevalonate metabolites could be recognized by Vγ9Vδ2 T cells as tumor phosphoantigens (10, 14), suggesting that the anticancer potency of Vγ9Vδ2 T cells might depend on intracellular IPP/ApppI levels in NBP–treated tumors. However, there is no in vivo evidence that a NBP can induce IPP/ApppI accumulation in tumors. In addition, the reasons why IPP/ApppI-producing tumors may become vulnerable to Vγ9Vδ2 T cells in vivo remain unexplored. In the present study, the correlation between the potency of Vγ9Vδ2 T cells to kill cancer cells and intracellular IPP/ApppI levels in ZOL-treated breast cancer cells was investigated in vitro and in vivo.
MATERIALS AND METHODS

Drugs, Cell Culture, and Animals

Unlabeled and carbon 14 (14C)-labeled ZOL was provided by Novartis Pharma AG (Basel, Switzerland). Recombinant IL-2 was provided by Novartis Pharmaceuticals Ltd (Horsham, UK). Sterile stock bisphosphonate solutions were prepared in PBS (pH 7.4; Invitrogen, Paisley, UK).

Five-week-old female SCID-NOD immunocompromised mice were purchased from Charles River Laboratories (Wilmington, MA). All procedures involving animals, including their housing and care, the method by which they were killed, and experimental protocols were conducted in accordance with a code of practice established by the local ethical committee (CREEA: Comité Régional d’Éthique pour l’Expérimentation Animale).

Blood was donated by healthy volunteers or obtained from the Blood Transfusion Center (Etablissement Français du Sang, Beynost, France). Human PBMCs were isolated after Ficoll-Paque (Amersham Biosciences, Uppsala, Sweden) density gradient centrifugation. The Vγ9Vδ2 T cells were expanded by exposing PBMCs to 10-µM ZOL plus 100-U IL-2 for 14 days. Populations of Vγ9Vδ2 T cells were purified by positive selection of TCR γδ cells using immunomagnetic cell sorting (AutoMACS® Pro Separator, Miltenyi Biotec, Bergisch Gladbach, Germany).

Human breast cancer cell lines (T47D, MCF-7, BT-474, ZR-75-1, MDA-MB-231, MDA-MB-435, MDA-MB-435s) were obtained from the American Type Culture Collection (ATCC)-LGC Promochem (Molsheim, France) and used within 6 months. All cell lines were authenticated using short tandem repeat analysis. The human estrogen-receptor (ER)-negative B02 breast cancer cell line, a subpopulation of MDA-MB-231, was prepared as previously described (28). PBMCs and ZR-75-1 breast cancer cells were cultured in RPMI-1640 medium (ATCC) supplemented with 10% fetal bovine serum (Invitrogen, Paisley, UK) and penicillin/streptomycin (Invitrogen). BT-474 breast cancer cells were cultured in HybriCare medium (ATCC) supplemented with 1.5 g/L NaHCO3, 10% fetal bovine serum and...
penicillin/streptomycin (Invitrogen). Other breast cancer cell lines were cultured in DMEM medium (Invitrogen) supplemented with 10% fetal bovine serum and penicillin/streptomycin.

**In Vitro and In Vivo Vγ9Vδ2 T-Cell Expansion, and IFN-γ Measurement**

PBMCs were seeded in 24-well plates (1×10^6 cells/well) then treated with vehicle (PBS), or ZOL (1, 10, or 100 µM) ± IL-2 100U/mL for 7, 14, 21, and 28 days. IL-2 was renewed at day 4 and then every 3 days. In vivo expansion of Vγ9Vδ2 T cells was performed in SCID-NOD mice. PBMCs (3.5×10^7) were inoculated intraperitoneally, and mice were treated with vehicle or ZOL (3, 10, or 30 µg/kg), intraperitoneally, and 10,000-U IL-2. Mice then received IL-2 plus vehicle or ZOL every 2 days for 14 days. On day 14, mice were sacrificed and peritoneal cells collected for analysis of Vγ9Vδ2 T cells. Percentages of Vγ9Vδ2 T cells in PBMCs and peritoneal cells were determined by flow cytometry (FACScan, Becton Dickinson, Franklin Lakes, NJ) after immunostaining with anti–CD3-PE (BD Pharmingen™, San Diego, CA) and anti–Vδ2-TCR-FITC antibodies (Beckman Coulter, Marseilles, France). Human IFN-γ in the serum from mice was measured using a commercial ELISA kit (Biosource; Nivelles, Belgium).

**IPP/ApppI Analysis**

ZOL-induced IPP/ApppI production was measured in PBMCs and breast cancer cells in vitro and in subcutaneous tumors in vivo. PBMCs (3×10^6) were exposed to PBS or ZOL (1, 10, or 100 µM) plus 100U/mL IL-2, then cultured on 6-well plates for 1, 2, 4, 7, 11, and 14 days. T47D, MCF-7, and B02 breast cancer cells were seeded in 6-well plates at 1×10^6 cells/well overnight. Cells were pulse-treated with 25-µM ZOL for 1h, then incubated without drug for 0–42 hours. Cells were then scraped, washed in PBS and extracted using ice-cold acetonitrile (300 µL) and water (200 µL) containing 0.25-mM NaF and Na_3VO_4 to prevent degradation of IPP and ApppI. Tumor xenografts were collected from mice after sacrifice and snap-frozen in liquid nitrogen, pulverized, and extracted using ice-cold acetonitrile. IPP and ApppI in cell extracts were quantified by high-performance liquid chromatography negative ion electrospray ionization mass spectrometry (HPLC-ESI-MS) (13).
Cellular Uptake Assay for ZOL

T47D, MCF-7, and B02 cells were seeded overnight to 10-cm Petri dishes at $4 \times 10^6$ cells/dish then treated with $^{14}$C-labeled ZOL for 1h. Cells were then rinsed with PBS, scraped, and extracted with acetonitrile and water. Extracts were separated by centrifugation (14,000×g, 2 minutes). Precipitates were analyzed for total protein content by a modified Bradford procedure. The soluble acetonitrile/water extracts were evaporated and redissolved in 120-µL Milli-Q water, then mixed with OptiPhase HiSafe3 scintillation cocktail (PerkinElmer Wallac, Turku, Finland) and evaluated by liquid scintillation (TriLux Microbeta™, PerkinElmer Wallac).

HMG-CoA Analysis by Western Blotting

T47D, MCF-7, and B02 cells were treated with vehicle (0.5% DMSO) or 0.3-µM lovastatin for 24h and then lysed in 100-µL cell lysis buffer [1% (vol/vol) Nonidet P-40 in 0.05M Tris-HCL containing 0.15-M NaCl, 1-mM EDTA, 0.05-M NaF and a cocktail of protease inhibitors (1-mM PMSF, 1-µg/ml aprotinin, 1-µg/ml leupeptin and 1-mM Na$_3$VO$_4$)]. For each sample, 50-µg protein was used for SDS-polyacrylamide gel electrophoresis, and transferred onto a PVDF membrane (Invitrogen), which was then washed with PBS/0.1% Tween and incubated overnight at 4°C with a rabbit polyclonal anti-HMG-CoA Ab (1/500 in 5% nonfat milk; Upstate, Lake Placid, NY). After washing, membranes were incubated with HRP-conjugated anti-rabbit Ab (GE Healthcare, Little Chalfont, UK) and developed using Western Lightning™ Chemiluminescence (PerkinElmer LAS Inc., Boston, MA). As a control, washed membranes were incubated with HRP-conjugated anti-mouse Ab (GE Healthcare) then developed as before. Densitometric analysis of blots was carried out using Quantity One software (Bio-Rad Laboratories, Hercules, CA).

Cytotoxicity Assay

T47D, MCF-7, and B02 breast cancer cells had different growth rates in culture. For cytotoxicity assessments, it was therefore necessary to adjust the cell number at the beginning of the experiment in order to have cell monolayers at a similar confluence at the
time of the coculture with Vγ9Vδ2 T cells. In this respect, T47D (2×10^4 cells/well), MCF-7 (2×10^4 cells/well), and B02 (7×10^3 cells/well) breast cancer cells were incubated overnight, then treated for 1h with vehicle (PBS) or ZOL (1–25 µM). Cell monolayers were then washed; 18h later, breast cancer cells were cocultured with or without purified Vγ9Vδ2 T cells (cancer cell:Vγ9Vδ2 T-cell ratio was 1:12.5 or 1:25) for 4h or 24h. Viability was assessed by MTT assay.

Experimental Tumorigenesis

Five-week-old female SCID-NOD mice were injected subcutaneously in the flank with 5×10^6 B02 or T47D cells in 100-µL PBS. For the estrogen receptor-positive T47D cells, host mice were implanted with subcutaneous 60-day-release pellets containing 1.7-mg 17β-estradiol (Innovative Research of America, Sarasota, FL) 4 days before tumor-cell inoculation. Four weeks later, when B02 and T47D tumors had reached a volume of 50 mm^3, mice were randomly assigned to 4 treatment groups (n = 6–7 mice/group): placebo (PBS); ZOL at 30 µg/kg body weight; human PBMCs (3.5×10^7) injected intraperitoneally plus 10,000-U IL-2 administered alone or with 30-µg/kg ZOL. In the relevant groups, IL-2 and ZOL in 0.5-mL PBS were administered intraperitoneally every 2 days for 14 days. Tumor size was calculated by external measurement of the width (m_1) and length (m_2) of s.c. tumor xenografts using a Vernier caliper. Tumor volume (TV) was calculated using the equation TV=(m_1^2×m_2)/2. At the end of the protocols, mice were sacrificed and tumors collected for immunohistochemistry and real-time polymerase chain reaction (PCR).

Immunohistochemistry

Tumors were embedded in Tissue-Tek (Sakura, Zoeterwoude, The Netherlands), snap-frozen in liquid nitrogen-cooled isopentane, and stored at –70°C. Frozen 7 µm sections were cut in a cryostat, air-dried, and fixed in cold acetone. After washing in PBS, tissue sections were preincubated with 1% goat serum. For detection of Vγ9Vδ2 T cells, tumor sections were incubated with a FITC-conjugated anti-human TCR δ2 monoclonal Ab, diluted 1:20 in PBS/1% BSA/0.3% Triton X-100. Cell nuclei were stained with DAPI (Roche,
Mannheim, Germany). Tissue sections were rewashed and visualized by fluorescence microscopy (Axioskop 40; Zeiss). To measure tumor-cell proliferation, tumor sections were incubated with a rabbit polyclonal anti–Ki-67 Ab (Abcam, Cambridge, UK). Experiments were carried out as previously described (28). The mitotic index was expressed as percentage of Ki-67–positive nuclei.

**Real-time PCR**

Total RNA was extracted from T47D and B02 tumors and infiltrating Vγ9Vδ2 T cells. Tumors were homogenized with a Polytron® device (Kinematica, Littau Lucerne, Switzerland). RNA was extracted using the nucleospin RNA II kit (Macherey-Nagel, Hoerdt, France) followed by DNase digestion (Macherey-Nagel, Hoerdt, France). Samples of total RNA (1.5-5 µg) were reverse-transcribed using Superscript™ II (Invitrogen). Real-time PCR was performed (IQ™ SYBR Green, Bio-Rad) with primers specific for the human housekeeping gene L32 (100 bp), a ribosomal protein used as an internal standard: 5′caaggacgtggaagtgtgt; 3′cagctcttttcacgatggc, and human TCR Vδ2 (162 bp): 5′caaaaccatccgtttttgct; 3′cttgacagcattgtacttcc. We confirmed the linear range of amplification for all primers and products. Real-time reverse transcription (RT)-PCR was carried out using the Mastercycler® EP system (SYBR Green; Realplex2, Eppendorf). Amplimers were quantified in triplicate samples in the pool of 3 independent tumors for each gene and normalized to corresponding L32 values.

**Transwell Migration Assay**

T47D, MCF-7, and B02 breast cancer cells were seeded overnight to 6-well plates at 10⁶ cells/well. The cells were then treated for 1h with PBS or 25-µM ZOL ± 10-µM lovastatin. Cells were then washed twice with PBS and cultured for 18h in DMEM + 0.25% BSA. The conditioned medium from treated or untreated breast cancer cell lines was collected and centrifuged for use in migration assays. Migration assays were performed in 24-well Transwell plates (Corning Costar, Corning, NY) with 5-µm pore polycarbonate membrane. Before migration assays, Vγ9Vδ2 T cells were enriched by PBMC culture with 10-µM ZOL.
plus 100U IL-2 for 10–14 days. Purified populations of Vγ9Vδ2 T cells obtained by positive selection of TCR γδ were resuspended at 3×10^6 cells/100 µL in assay buffer. A cell suspension (100 µL) was placed in the upper chamber of the Transwell inserts, and 600 µL of conditioned medium from breast cancer cell lines, plus 1 µM ApppI or 0.5 µM IPP were placed in the lower chamber. After incubation for 4 or 24h at 37°C, cells from the lower chamber were counted. Basal cell culture medium was used as a control to measure random migration of Vγ9Vδ2 T cells in the absence of chemoattractant. The control was set to 100% and the results obtained in the presence of a chemoattractant were expressed as a percentage of the control.

Human cytokines released from vehicle- and ZOL-treated T47D and B02 cells were detected using RayBio Human Cytokine Ab Array C series 1000, designed to detect 120 cytokines (Ray Biotech, Norcross, GA). The array membranes were incubated overnight with conditioned medium (1.2 mL/1.2 mg protein) from cultured T47D and B02 cells treated or untreated with ZOL. Detection of immunoreactive spots was carried out using an enhanced chemiluminescence detection system.

Human chemokines IL-8, IL-6, and monocyte chemoattractant protein-1 (MCP-1; eBioscience) released from vehicle- and ZOL-treated T47D, MCF-7, and B02 cells were measured by ELISA.

Statistical Analysis

All data were analyzed using StatView software (version5.0; SAS Institute Inc, Cary, NC). Pairwise comparisons were carried out by performing a nonparametric Mann-Whitney U test. P values less than .05 were considered statistically significant. All statistical tests were two-sided.
RESULTS AND DISCUSSION

ZOL Induces Human Vγ9Vδ2 T-Cell Expansion In Vitro and In Vivo

ZOL stimulated, in a time- and dose-dependent manner, expansion of Vγ9Vδ2 T cells from human PBMCs, peaking at day 14 with a 10-µM ZOL concentration (Figure 1A). These results are in accordance with previous reports showing that NBPs stimulate proliferation of Vγ9Vδ2 T cells in vitro (29). In addition, mass spectrometry revealed rapid IPP accumulation in PBMCs after 1–2 days of ZOL treatment (Figure 1B), whereas ApppI was detected after 2–4 days (Figure 1C).

No reactivity of murine γδ T cells to phosphoantigens such as IPP or ApppI has yet been demonstrated and human γδ T cells do not recognize cells from animals other than humans (11,30). Thus, murine PBMCs treated with an amino-bisphosphonate do not activate murine or human γδ T cells. The effect of ZOL on Vγ9Vδ2 T-cell expansion was therefore studied in vivo after intraperitoneal injection of human PBMCs into SCID-NOD mice and ZOL+IL-2 treatment. ZOL stimulated expansion of Vγ9Vδ2 T cells such that they comprised up to 16% of T lymphocytes, versus 2.7% for IL-2 alone (Figures 1D and 1E). This is explained by the fact that ZOL is internalized by antigen-presenting cells from human PBMCs where it induces the intracellular accumulation of IPP/ApppI and subsequent activation of Vγ9Vδ2 T-cell expansion (12), making our animal model most suitable for studying the role of ZOL in cancer immunotherapy in vivo. Moreover, although ZOL was administered more frequently in our model than the current clinical dosing regimen in humans, the dose given (30 µg/kg) was lower than the dosing in humans (100 µg/kg), supporting the clinical relevance of our findings.

ZOL Induces IPP/ApppI Production in Human Breast Cancer Cells In Vitro

Prior studies showed the intracellular accumulation of IPP and ApppI in NBP-treated cancer cell lines in vitro (10, 27). In our study, ZOL-induced IPP/ApppI production was quantified in a series of human breast cancer cell lines having a luminal (ER-positive and/or
progesterone receptor [PR]-positive and Ki-67-positive) or basal (ER negative and PR negative and epidermal growth factor receptor-2 [HER2]-negative) molecular subtype. Mass spectrometry analysis showed that IPP/Apppl levels varied substantially among breast cancer cell lines after ZOL treatment (Figure 2A). Specifically, 3 out of the 4 luminal breast cancer cell lines studied (T47D, MCF-7, BT-474, ZR-75-1) produced IPP/Apppl, whereas none (0/4) of the basal breast cancer cell lines tested (MDA-MB-231, B02, MDA-MB-435 and MDA-MB-435s) produced these phosphoantigens. In addition, we found that IPP/Apppl accumulation in ZOL-treated T47D cells was time-dependent, with IPP reaching a maximum concentration at 12h (1,052 pmol/mg protein) and Apppl peaking at 24h (1,302 pmol/mg protein) (Figure 2B). These results were in line with our previous findings showing that the basal MCF-10A and MDA-MB-436 breast cell lines produced low levels of IPP/Apppl after ZOL treatment (27). The higher IPP/Apppl production in luminal versus basal breast cancer cell lines is, to our knowledge, a novel observation.

Three breast cancer cell lines having either a high (T47D), medium (MCF-7), or low (B02) production of phosphoantigens were then selected for subsequent experiments. Fluid-phase endocytosis is the major route by which NBPs are internalized in macrophages and osteoclasts (31). Variations in endocytosis between the cancer cell lines might explain differences in IPP/Apppl production induced by ZOL. Uptake of 14C-labeled ZOL was therefore measured in T47D, MCF-7, and B02 cells. Uptake was similar in T47D and MCF-7 cells, but 3-fold lower in B02 cells (Figure 2C). Additionally, we found a strong correlation between HMG-CoA reductase expression, a mevalonate pathway enzyme upstream of FPP synthase, and ZOL-induced IPP/Apppl accumulation (Figure 2D). As shown by Western blotting, lovastatin (an HMG-CoA reductase inhibitor) substantially increased HMG-CoA protein levels in ER-positive T47D and MCF-7 cells (Figure 2D). By contrast, HMG-CoA reductase was undetectable, and only a faint protein band was observed in lovastatin-treated ER-negative B02 cells (Figure 2D). These results agree with data reported by Borgquist et al. (32), who showed that high HMG-CoA reductase expression correlated positively with ER expression in tumor tissue from patients with breast cancer. Altogether, these results
demonstrate that IPP/ApppI production in breast cancer cells depends on cellular uptake of NBP and on the activity of the mevalonate pathway. Moreover, we suggest that luminal neoplastic mammary epithelial cells might be more sensitive to bisphosphonate therapy than basal neoplastic mammary epithelial cells.

Vγ9Vδ2 T-Cell Cytotoxicity Correlates with ZOL-induced IPP/ApppI Accumulation in Breast Cancer Cells In Vitro and In Vivo

The antitumor potency of human Vγ9Vδ2 T cells against ZOL-treated breast cancer cell lines was first examined in vitro (Figures 3A–3F). Incubation for 1h with ZOL (1–25 µM) did not affect survival of T47D, MCF-7, and B02 cells (Figures 3A, 3C, and 3E, respectively), whereas these bisphosphonate concentrations did induce intracellular IPP/ApppI accumulation in T47D and MCF-7 cells (Figures 2A-B). Coculture of ZOL-treated T47D cells with purified Vγ9Vδ2 T cells led to dose-dependent cancer cell death, which was statistically significant with ZOL at concentrations as low as 1 µM for 1h (Figure 3B). Additionally, Vγ9Vδ2 T cells caused ZOL-treated MCF-7 cell death; concentrations of ZOL ≥10 µM were required to “prime” MCF-7 cells for Vγ9Vδ2 T-cell–mediated cytotoxicity (Figure 3D). In sharp contrast, Vγ9Vδ2 T cells were not cytotoxic against ZOL-treated B02 cells (Figure 3F).

Although human cancer cell lines exhibit susceptibility to Vγ9Vδ2 T-cell-mediated cytotoxicity upon NBP treatment, the in vitro antitumor activity of Vγ9Vδ2 T cells against tumor cells treated with NBPs varies greatly (10,11,15). For example, Vγ9Vδ2 T cells fail to efficiently kill a variety of human renal (ACHN, Caki-2, A-704) and gastric (MKN45, MKN74) cancer cell lines after NBP pretreatment (11). Here, our results strongly suggest that ZOL-induced IPP/ApppI accumulation in breast cancer cells was responsible for Vγ9Vδ2 T cell-mediated cytotoxicity. In this respect, co-treatment of T47D cells with ZOL+lovastatin almost completely eliminated IPP/ApppI phosphoantigen production by these cells and substantially reduced Vγ9Vδ2 T cell cytotoxicity (supplementary data; Figure S1). It is therefore most conceivable that the lack of cytotoxicity of Vγ9Vδ2 T cells against some cancer cell lines is
attributable to a low intracellular accumulation of phosphoantigens upon NBP treatment of cancer cells, likely because of the low activity level of the mevalonate pathway.

We next conducted in vivo experiments to examine the contribution of IPP/ApppI accumulation in breast tumors to Vγ9Vδ2 T-cell cytotoxicity. Earlier experimental in vivo studies used human Vγ9Vδ2 T cells expanded in vitro and then purified before inoculation (15, 18, 19, 20). Here, we showed that ZOL + IL-2 treatment induced Vγ9Vδ2 T-cell expansion from human PBMCs injected intraperitoneally into SCID-NOD mice (Figure 1E). In the clinic, it has been recently shown that the treatment of advanced breast cancer patients with ZOL and low-dose IL-2 triggers Vγ9Vδ2 T cell amplification in vivo (24). We therefore chose to use this therapeutic strategy to treat tumor-bearing animals. SCID-NOD mice bearing subcutaneous breast cancer xenografts were inoculated with human PBMCs, with expansion of Vγ9Vδ2 T cells in vivo (Figure 4). Treatment with ZOL alone or PBMC+IL-2 did not inhibit subcutaneous tumor growth (Figures 4A and 4B). In contrast, PBMC + IL-2 + ZOL completely blocked growth progression of T47D tumors, compared to that observed in placebo-treated animals (P<.01; Figure 4A). In addition, in situ immunodetection of the proliferation marker Ki-67 nuclear antigen in T47D tumors from mice treated with PBMC + IL-2 + ZOL showed drastic reduction in proliferative index versus tumors from placebo-treated mice (Figure 4C). However, this treatment did not inhibit growth or proliferative index of B02 subcutaneous tumors (Figures 4B and 4D). Of note, Vγ9Vδ2 T-cell infiltrates were detected in T47D but not B02 tumors from mice treated with PBMC+IL-2+ZOL (Figures 4E, 4F, and 4G), as judged by both RT-PCR and immunohistochemistry. Moreover, serum levels of human IFN-γ were high in T47D-tumor-bearing mice treated with PBMCs + IL-2 + ZOL (942 ± 240 pg/mL, n = 5) and moderate in animals treated with PBMCs + IL-2 (563 ± 370 pg/mL, n = 5), whereas the cytokine was not detected in animals treated with placebo or ZOL alone, demonstrating that Vγ9Vδ2 T cells were activated in vivo.

Because Vγ9Vδ2 T cells infiltrated subcutaneous T47D tumor xenografts in mice treated with PBMC+IL-2+ZOL (Figures 4E and 4G), IPP/ApppI was measured in tumor
extracts by mass spectrometry. As expected, IPP and Apppl were barely detectable in T47D tumors from placebo-treated mice (Figure 5A), whereas IPP levels were substantial 24h after the last ZOL dose in mice treated with PBMC + IL-2+ ZOL (3.7 pmol; 82 nM) (Figure 5B). By contrast, Apppl remained undetectable at 24h. Conversely, lower IPP levels were detected at 48h after the last ZOL dose in PBMC + IL-2 + ZOL-treated mice (0.7 pmol; 16 nM), whereas Apppl levels were measured (0.11 pmol; 2.5 nM) (Figure 5C). Of note, IPP and Apppl were also detected in T47D tumor extracts from mice treated with ZOL alone. In contrast, ZOL-induced IPP/Apppl was not detected in B02 tumor xenografts (not shown).

These data, to the best of our knowledge, show for the first time that subcutaneous tumors can produce IPP/Apppl after ZOL administration, demonstrating the uptake of the NBP into tumor cells in vivo. If IPP/Apppl had been produced by tumor-infiltrating cells (eg, macrophages), we would have expected IPP/Apppl to also be produced in B02 tumors, contrary to what we observed in vivo. It is interesting that Guenther et al. (33) recently reported, utilizing a mouse xenograft model for plasmacytoma, that the treatment of animals with ZOL leads to accumulation of unprenylated Rap1A (a surrogate marker of the inhibitory effect of NBP on the mevalonate pathway) in plasma cell tumors ex vivo. These findings (33), along with our present data, provide evidence for a direct uptake of ZOL in tumor cells in vivo. However, in our breast cancer model, ZOL treatment without PBMC + IL-2 (ie, without Vγ9Vδ2 T-cell expansion) did not inhibit tumor growth, demonstrating the important role of human Vγ9Vδ2 T cells in the antitumor activity of NBPs.

**IPP and Apppl Released by Breast Cancer Cells are Chemotactic Factors for Vγ9Vδ2 T Cells**

In our model, ZOL was able to induce recruitment of Vγ9Vδ2 T cells to T47D tumors. We therefore investigated the ability of breast cancer cells treated in vitro with ZOL to promote Vγ9Vδ2 T-cell migration. Using conditioned medium from untreated breast cancer cell lines (T47D, MCF-7, B02) in Transwell® migration assays, we showed significantly enhanced migration of Vγ9Vδ2 T cells after 4h (not shown) and 24h (Figure 6A; P < .05 and
Migration of Vγ9Vδ2 T cells was much higher with conditioned medium from ZOL-treated T47D and MCF-7 breast cancer cells (Figures 6B and 6C). Additionally, conditioned medium from T47D cells treated with ZOL+lovastatin resulted in less migration of Vγ9Vδ2 T cells (Figure 6C). To clarify whether IPP/ApppI acted as migration factors for Vγ9Vδ2 T cells, we used mass spectrometry to measure IPP levels in conditioned medium from ZOL-treated breast cancer cell lines (T47D, MCF-7, B02). We found substantial IPP levels in conditioned medium from ZOL-treated T47D and MCF-7 cells, but not B02 cells (data not shown). Moreover, 0.5-µM IPP or 1-µM ApppI significantly stimulated γδ T-cell migration (Figure 6C). Because ApppI can be converted into IPP on hydrolysis by nucleotide pyrophosphatases (14), it also may be processed into IPP for subsequent recognition by the Vγ9Vδ2 TCR. The cytokines IL-6, IL-8, and MCP-1 are also γδ T-cell chemoattractants (30). However, we found no differences in these or any other cytokines in a human cytokine antibody array of conditioned medium from untreated or ZOL-treated T47D, MCF-7, and B02 cells (Figure 6D). Although B02 cells produced a greater range of cytokines than T47D cells, cytokine profiles were similar for ZOL-treated or untreated cells (Figure 6D). By ELISA, untreated B02 cells (control) produced more IL-8 and MCP-1 compared with T47D or MCF-7 control cells (Figure 6E); IL-6 was detected in B02 cells only.

In conclusion, our study shows for the first time that ZOL induces the production of IPP/ApppI phosphoantigens by breast tumors in vivo which, in turn, promote chemotaxis and cytotoxicity of Vγ9Vδ2 T cells. These results provide some support for an adjuvant role of NBPs in breast cancer. There is now clinical evidence that adding ZOL to endocrine therapy improves disease-free survival in estrogen-responsive early breast cancer (4,5). ZOL has also been shown to durably activate γδ T cells in patients with breast cancer (25). The immunomodulating role of NBPs on human γδ T cells might explain, at least in part, the anticancer effects of ZOL observed in these adjuvant clinical trials. Overall, our findings suggest that cancers producing high IPP/ApppI levels after ZOL treatment are most likely to benefit from Vγ9Vδ2 T-cell–mediated immunotherapy.
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FIGURE LEGENDS

Figure 1. ZOL-induced Vγ9Vδ2 T-cell expansion in vitro and in vivo, and IPP/ApppI production in human PBMCs. (A) Peripheral blood mononuclear cells (PBMCs) were treated with 1, 10, or 100 µM ZOL plus 100U/mL IL-2, or with IL-2 alone. Vγ9Vδ2 T-cell proliferation was analyzed by flow cytometry after 0–28 days culture. Data are expressed as mean ± SD of triplicate cultures from a representative donor. Accumulation of IPP (B) and ApppI (C) in PBMCs after 1–14 days culture. Detection limit 1 pmol/mg protein. Data are mean ± SD of duplicate cultures (representative donor). Dot plot of flow cytometry from peritoneal cells of SCID-NOD mice receiving PBMCs and 10,000-U IL-2 alone (D) or with 30-µg/kg ZOL (E), both every 2 days for 14 days. Plots are representative of 5 mice. ApppI, triphosphoric acid I-adenosin-5'-yl ester 3-(3-methylbut-3-enyl) ester; IL2, interleukin; IPP, isopentenyl pyrophosphate; nd, not detected; ZOL, zoledronic acid.

Figure 2. IPP/ApppI accumulation induced by ZOL depends on activity of the mevalonate pathway and cellular uptake of ZOL. (A) Breast cancer cells were treated with 25-µM ZOL for 1h, then cultured without drug for 18h (mean ± SD, n=12–21). Detection limit 1 pmol/mg protein. (B) T47D cells were treated with 25-µM ZOL for 1h and samples collected 0–42h after drug removal (mean ± SD, n=3). (C) Cellular uptake of 14C-labeled ZOL 1h after 25-µM ZOL treatment (mean ± SD, n=3). (D) Bands of HMG-CoA reductase (HMG-CoAR) and tubulin by Western blotting in cancer cell lines after treatment with 0.5% DMSO (basal) or 0.3-µM lovastatin for 24h. Band density of HMG-CoAR is normalized by tubulin. ApppI, triphosphoric acid I-adenosin-5'-yl ester 3-(3-methylbut-3-enyl) ester; 14C, carbon-14; IPP, isopentenyl pyrophosphate; ZOL, zoledronic acid.

Figure 3. ZOL-induced IPP/ApppI accumulation in breast cancer cells correlates with Vγ9Vδ2 T-cell–mediated cancer cell death. T47D, MCF-7, and B02 cells were pretreated with control (CTR; PBS) or ZOL 1–25 µM for 1h, then cultured without drug for 18h. After
incubation, cells were cocultured without (A, C, E) or with (B, D,F) purified Vγ9Vδ2 T cells for 24h. The ratio of cancer cells to Vγ9Vδ2 T cells was 1:12.5. (A–F) are representative of 3 independent experiments (mean±SD). *P<.05; **P<.01. Apppl, triphosphoric acid I-adenosin-5'-yl ester 3-(3-methylbut-3-enyl) ester; IPP, isopentenyl pyrophosphate; ZOL, zoledronic acid.

**Figure 4.** Cytotoxic effect of Vγ9Vδ2 T cells against T47D and B02 breast cancer subcutaneous tumor xenografts. Mice were inoculated subcutaneously with T47D or B02 cells. Four weeks after tumor cell inoculation, when tumors became palpable, mice were engrafted with peripheral blood mononuclear cells (PBMCs) from healthy human adult blood donors. Additionally, mice were treated with IL-2 ± ZOL (30 µg/kg) every 2 days for 2 weeks. T47D (A) and B02 (B) tumor growth was determined at weeks 4 and 6 after tumor cell inoculation, by measurement of the tumor volume with a Vernier caliper. T47D (C) and B02 (D) Ki-67 indices were assessed by immunohistochemistry. The relative expression of Vγ9Vδ2 T cells in T47D (E) and B02 (F) tumors was assessed by RT-PCR. (G) Presence of Vγ9Vδ2 T cells in T47D tumors from the placebo and PBMC+IL-2+ZOL groups. Tumor sections were stained with TCR Vδ2 (green) and DAPI (nuclei, blue). Results are mean ± SD of 6–7 mice per group. ns, not significant; *P<.05 and **P<.01, compared to placebo. IL-2, interleukin-2; ZOL, zoledronic acid.

**Figure 5.** Mass spectrometry analysis of IPP and Apppl in T47D breast cancer subcutaneous tumor xenografts.

Identification of IPP and Apppl in T47D tumors of placebo- and ZOL-treated mice was performed by high-performance liquid chromatography negative ion electrospray ionization mass spectrometry. Selected reaction monitoring chromatograms are shown. They correspond to tumor extracts obtained from placebo-treated mice (A) and animals that received PBMC + IL-2 + ZOL whose tumors were collected 24h (B) and 48h (C) after the last
ZOL injection. Similar chromatograms were obtained with tumor extracts from mice treated with ZOL alone. Tumor extracts were spiked with 1.35-pmol IPP and 0.11-pmol Apppl. IPP and Apppl chromatograms are drawn on the same scale (standards). Apppl, triphosphoric acid l-adenosin-5'-yl ester 3-(3-methylbut-3-enyl) ester; IL-2, interleukin; IPP, isopentenyl pyrophosphate; PBMC, peripheral blood mononuclear cells; ZOL, zoledronic acid.

**Figure 6. IPP and Apppl induce chemotactic migration of Vγ9Vδ2 T cells.** Purified Vγ9Vδ2 T cells isolated from ZOL-stimulated peripheral blood mononuclear cells (PBMCs) by positive selection of T-cell receptor (TCR)-γδ cells were tested in a Transwell assay for their capacity to migrate: (A) Toward conditioned medium (CM) from T47D, MCF-7, and B02 cell lines compared with assay medium alone; (B) Toward CM from ZOL-treated T47D, MCF-7, and B02 cells vs CM from untreated cells; or (C) Toward CM from ZOL-treated T47D cells ± lovastatin and 0.5-µM IPP or 1-µM Apppl added in the medium, or compared with assay medium alone. Data represent mean percentage migration from 2 independent experiments ± SD. *P<.05; **P<.01 and *** P<.001 using pairwise comparisons with the assay medium or CM from untreated cells. (D) Detection of cytokines produced in the supernatant by cultured, untreated (control; CTR), and ZOL-treated T47D and B02 cells using two human cytokine antibody array membranes (1 & 2) designed to detect altogether 120 human cytokines. (E) IL-6, IL-8, and MCP-1 secretion in CM of T47D, MCF-7, and B02 cells, using ELISA. Abbreviation: CM, conditioned medium. Apppl, triphosphoric acid l-adenosin-5'-yl ester 3-(3-methylbut-3-enyl) ester; IL, interleukin; IPP, isopentenyl pyrophosphate; ns, not significant; ZOL, zoledronic acid.
Figure 1

A

B

C

D

E

T lymphocytes

Vc9Vα2 T

16% Vc9Vα2 T

2.7% Vc9Vα2 T
Figure 3

- γδ T cells

- γδ T cells

T47D

MCF-7

BO2

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Figure 4

Tumor cells → Human PBMCs

4 Weeks → 2 Weeks

± ZOL IL2

RT-PCR IHC

A. T47D tumors

B. BO2 Tumors

C. Ki67 index (%)

D. Ki67 index (%)

E. Relative VSV-G2 expression

F. Relative VSV-G2 expression

G. Immunohistochemistry
Figure 5

Tumor cells → Human PBMCs → MS

4 Weeks → 2 Weeks

± ZOL IL2

T47D Tumors

Tumor volume (mm³) after 2 week treatment

Placebo ZOL PBMC + IL2 PBMC + IL2 + ZOL

ns ns

Standards

[Chemical structures and mass spectrometry graphs]

IPP, m/z 245 → 159
Apppl, m/z 574 → 408
Figure 6

A

Cell migration (% of control)

Control  T47D, CM  MCF-7, CM  BO2, CM

0  50  100  150  200  250

B

Cell migration (% of control)

T47D  MCF-7  BO2

-  +

ZOL  ZOL  ZOL

-  +

-  +

-  +

C

Cell migration (% of control)

Control  -ZOL  +ZOL  +ZOL  IPP  Apppl

-  +

-  +

-  +

-  +

D

T47D

CTR  ZOL

Angiogenin

MCP-1

GRO, GRO-α

E

Secreted cytokine (pg/ml)

IL-6  IL-8  MCP-1

T47D, CM  MCF-7, CM  BO2, CM

nd  nd  BO2, CM
High Phosphoantigen Levels in Bisphosphonate-Treated Human Breast Tumors Promote Vγ9Vδ2 T-Cell Chemotaxis and Cytotoxicity In Vivo

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