In-Vivo Phosphoantigen Levels in Bisphosphonate-Treated Human Breast Tumors Trigger $\gamma\delta\nu9\nu2$ T-Cell Antitumor Cytotoxicity through ICAM-1 Engagement.

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Relevance

Human γδ T cells straddle the border between innate and adaptive immunity and have potent anti-tumor activity. We recently provided in vivo evidence that bisphosphonate zoledronate induces accumulation of IPP/ApppI phosphoantigens in human breast tumors implanted in animals and that human Vγ9Vδ2 T cells infiltrate and inhibit growth of these tumors producing high IPP/ApppI levels, but not those expressing low IPP/ApppI levels. We found here similar findings using the bisphosphonate risedronate, and we showed that estrogen receptor (ER)-positive breast tumors are more likely to produce IPP/ApppI after bisphosphonate treatment compared with ER-negative breast tumors. Moreover, our study shows for the first time that the ability of risedronate and zoledronate to activate Vγ9Vδ2 T-cell anticancer activity not only depends on IPP/ApppI accumulation in ER-positive tumors but also on expression of tumor cell surface receptor ICAM-1, which triggers the recognition of bisphosphonate-treated breast cancer cells by Vγ9Vδ2 T cells in vivo.

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Abstract:

Purpose: Nitrogen-containing bisphosphonates (N-BPs) such as zoledronate (ZOL) and risedronate (RIS) exhibit antitumor effects. They block the activity of farnesyl pyrophosphate synthase in the mevalonate pathway, leading to intracellular accumulation of mevalonate metabolites (IPP/ApppI) which are recognized as tumor phosphoantigens by Vγ9Vδ2 T cells. However, mechanisms responsible for Vγ9Vδ2 T-cell recognition of N-BP-treated tumors producing IPP/ApppI remain unclear.

Methods: The effects of N-BPs on Vγ9Vδ2-T cell expansion and anticancer activity were evaluated in vitro and in animal models of human breast cancers. The modalities of recognition of breast tumors by Vγ9Vδ2-T cells in N-BP-treated animals were also examined.

Results: We found a strong correlation between Vγ9Vδ2-T cell anticancer activity and intracellular accumulation of IPP/ApppI in RIS-treated breast cancer cells in vitro. Additionally, following RIS treatment of immunodeficient mice bearing human breast tumors, human Vγ9Vδ2 T cells infiltrated and inhibited growth of tumors that produced high IPP/ApppI levels, but not those expressing low IPP/ApppI levels. The combination of doxorubicin with a N-BP improved however Vγ9Vδ2 T-cell cytotoxicity against breast tumors expressing low IPP/ApppI levels. Moreover, Vγ9Vδ2 T-cell cytotoxicity in mice treated with RIS or ZOL did not only depend on IPP/ApppI accumulation in tumors but also on expression of tumor cell surface receptor ICAM-1, which triggered the recognition of N-BP-treated breast cancer cells by Vγ9Vδ2 T cells in vivo.

Conclusion: These findings suggest that N-BPs can have an adjuvant role in cancer therapy by activating Vγ9Vδ2 T-cell cytotoxicity in patients with breast cancer that produces high IPP/ApppI levels after N-BP treatment.

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Introduction

Bisphosphonates, especially nitrogen-containing bisphosphonates (N-BPs), are widely used to treat bone-loss disorders, such as postmenopausal osteoporosis and skeletal morbidity associated with malignant bone diseases, because they bind avidly to bone mineral where they inhibit osteoclast-mediated bone resorption (1). N-BPs [e.g., pamidronate, alendronate, risendronate (RIS), ibandronate, zoledronate (ZOL), minodronate] specifically interfere with farnesyl pyrophosphate synthase (FPPS), a key enzyme in the mevalonate pathway (2). As a consequence, the covalent attachment of isoprenyl chains to small GTPases is blocked, thereby inhibiting their intracellular localization and functions in osteoclasts. Moreover, the disruption of the mevalonate pathway by N-BPs results in the accumulation of isopentenyl pyrophosphate (IPP), which is then converted to a cytotoxic ATP analogue called Apppl [triphosphoric acid l-adenosin-5'-yl ester 3-(3-methylbut-3-enyl) ester] (2). Thus, N-BPs may exert their pharmacological effects on osteoclasts through the formation of IPP/Apppl and via the inhibition of prenylation of small GTPases.

In addition to their therapeutic activity in preserving bone tissue, there is extensive preclinical evidence that N-BPs have anticancer benefits, acting on cells other than osteoclasts, such as tumor cells and endothelial cells (2, 3). For example, the prenylation of small G proteins essential for cancer cell adhesion, migration and invasion is impaired (2). N-BPs also inhibit angiogenesis in vivo (3). Moreover, by blocking the release of bone-derived growth factors during osteoclast-mediated bone resorption, N-BPs can indirectly slow tumor cell proliferation and survival (2). Importantly, there is now clinical evidence that adding ZOL to endocrine therapy improves disease-free survival and overall survival of patients with estrogen-responsive early breast cancer in a low estrogen environment (i.e., following ovarian suppression therapy or in women with established menopause at diagnosis) (4-6). How ZOL mediates this antitumor activity remains however elusive.

Emerging data suggest that NBPes have immunomodulatory properties by stimulating the expansion of Vγ9Vδ2 T cells, a subset of human T cells that straddles the border between innate and adaptive immunity and exhibits anticancer activity (7). Evidence for the
stimulation of \( V_{\gamma}9V_{\delta}2 \) T cells by N-BPs was first found when increased numbers of \( \gamma\delta \) T cells were observed in patients who had flu-like acute-phase reactions after their first intravenous infusion of pamidronate (8). N-BPs are indeed internalized by peripheral blood mononuclear cells (PBMCs), such as monocytes and dendritic cells, where they inhibit the mevalonate pathway, leading to the intracellular accumulation of IPP which, in turn, activates \( V_{\gamma}9V_{\delta}2 \) T cells and the release of inflammatory cytokines [tumor necrosis factor-\( \alpha \) (TNF-\( \alpha \)) and interferon-\( \gamma \) (IFN-\( \gamma \))], thereby contributing to the acute-phase reaction (7,9,10). In addition, ApppI could represent an inactive storage form of phosphoantigen which would require conversion to IPP to activate \( V_{\gamma}9V_{\delta}2 \) T cells from PBMCs (11).

N-BPs also induce intracellular accumulation of IPP/ApppI in a wide variety of human tumor cell lines in vitro and these mevalonate metabolites can be sensed by \( V_{\gamma}9V_{\delta}2 \) T cells as tumor phosphoantigens (2,7). We recently provided in vivo evidence that ZOL induces IPP/ApppI accumulation in human breast tumors implanted subcutaneously in animals and that human \( V_{\gamma}9V_{\delta}2 \) T cells infiltrate and inhibit growth of these tumors producing high IPP/ApppI levels, but not those expressing low IPP/ApppI levels (12). However, mechanisms responsible for \( V_{\gamma}9V_{\delta}2 \) T-cell recognition of ZOL-treated tumors producing IPP/ApppI remain unclear.

The modalities of recognition of N-BP-treated tumor cells by \( V_{\gamma}9V_{\delta}2 \) T cells are likely \( \gamma\delta \) TCR-dependent for IPP (7), but may also involve additional cell-cell contacts through unrelated TCR cell surface receptors expressed by \( V_{\gamma}9V_{\delta}2 \) T cells such as lymphocyte function-associated antigen-1 (LFA1), CD6, and natural killer group 2D (NKG2D) (13). LFA1, CD6 and NKG2D interact with intercellular adhesion molecule-1 (ICAM-1), CD166, and MHC class I–related chains A/B (MICA/MICB), respectively, which are expressed by tumor cells (13). These cell surface receptors have been involved in mediating \( V_{\gamma}9V_{\delta}2 \)-T cell cytotoxicity against ZOL-treated tumor cells in vitro (13). However, there is no evidence of their engagement for recognition of ZOL-treated tumors by \( V_{\gamma}9V_{\delta}2 \) T cells in vivo. Another consideration is whether other N-BPs might exert in vivo immunomodulating effects similar to...
those we previously reported for ZOL (12). This question is of great importance since different N-BPs [ibandronate, RIS, alendronate] are used to prevent cancer treatment-induced bone loss in breast cancer, and clinical trials investigating their potential role in the adjuvant setting to prevent metastasis are ongoing (1,14). In this study, we address whether RIS can activate human Vγ9Vδ2 T cells \textit{in vitro} and in animal models of breast cancer. In addition, we examine cell surface receptors through which Vγ9Vδ2 T cells could recognize human breast tumors from mice treated with RIS or ZOL.
Materials and Methods:

Drugs. Nitrogen-containing bisphosphonates RIS, NE-58025, NE-58051 and NE-10790, and fluorescent RIS were obtained from Procter & Gamble (Mason, OH, USA). 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).

Human cells and cell culture. Blood donated from healthy volunteers or obtained from the Blood Transfusion Center (Etablissement Français du Sang, Beynost, France) was collected in heparin. Human PBMCs were isolated after Ficoll-Paque (Amersham Biosciences, Uppsala, Sweden) density gradient centrifugation, as previously described (12). The Vγ9Vδ2 T cells were expanded by exposing PBMCs to 10-μM RIS plus 100-U IL-2 for 14 days. Vγ9Vδ2T cells were purified by positive selection of TCR γδ cells using immunomagnetic cell sorting (12).

Human cell lines from breast cancer (T47D, MCF-7, BT-474, ZR-75-1) and cervical carcinoma (Hela) were obtained from the American Type Culture Collection (ATCC)-LGC Promochem (Molsheim, France) and used within 6 months. The human B02 breast cancer cell line, a subpopulation of MDA-MB-231, was prepared as previously described (15). All cell lines were authenticated using short tandem repeat analysis. ZR-75-1 cells were cultured in RPMI-1640 medium supplemented with 10% (v/v) fetal bovine serum and penicillin/streptomycin. BT-474 cells were cultured in Hybri-Care medium (ATCC) supplemented with 1.5 g/L NaHCO3, 10% (v/v) fetal bovine serum and penicillin/streptomycin. MCF-7 cells were cultured in EMEM medium (ATCC) supplemented with 0.01mg/ml bovine insulin (Invitrogen), 10% (v/v) fetal bovine serum and penicillin/streptomycin. Other cancer cell lines were cultured in DMEM medium (Invitrogen) supplemented with 10% fetal bovine serum and penicillin/streptomycin.
**Cellular Uptake Assay for RIS.** T47D, MCF-7, and B02 cells were seeded overnight to 10-cm Petri dishes at $3\times10^5$ cells/dish then treated with fluorescent-labeled FAM-RIS 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 measured at 445nm for the intensity of the fluorescence using a 96-well fluorescent plate reader and results were expressed as picomol/mg total protein.

**Animals.** Five-week-old female SCID-NOD immunocompromised mice were purchased from Charles River Laboratories (Wilmington, MA). Animals were maintained in a 12-h light-dark cycle and given free access to food and water. All procedures involving animals, including the method by which they were culled and experimental protocols were conducted in accordance with a code of practice established by the ethical committee of the University of Lyon.

**In Vitro and In Vivo Vy9Vδ2-T-Cell Expansion.** PBMCs were seeded in 24-well plates (1×$10^5$ cells/well) then treated with vehicle (PBS), or with bisphosphonates ZOL, RIS, NE-58025, NE-58051 and NE-10790 (10 μM) ± IL-2 100U/mL for 14 days. IL-2 was renewed at day 4 and then every 3 days. In vivo expansion of Vy9Vδ2 T cells was performed in SCID-NOD mice. Human PBMCs (3.5×$10^7$) were inoculated intraperitoneally and mice were treated with vehicle or RIS (10, 30, 150, 300 or 600 μg/kg), intraperitoneally, and 10,000-U IL-2. Mice then received IL-2 plus vehicle or RIS every other days for 14 days. On day 14, mice were sacrificed and peritoneal cells collected for analysis of Vy9Vδ2 T cells. Percentages of Vy9Vδ2 T cells in PBMCs and peritoneal cells were determined by flow cytometry, as previously described (12). Human IFN-γ in the serum from mice was measured using a commercial ELISA kit (Biosource; Nivelles, Belgium).
**Vy9Vδ2 T-Cell-mediated Cytotoxicity Assay.** Human T47D, MCF-7, BT-474, ZR75-1 (2×10^4 cells/well) and B02 (7,000 cells/well) breast cancer cells were incubated overnight, then treated for 1h with vehicle (PBS) or RIS (1–25 μM). Cell monolayers were then washed; 18h later, human cancer cells were cocultured with or without purified Vy9Vδ2 T cells (cancer cell:Vy9Vδ2 T-cell ratio was 1:12.5) for 4h or 24h. Viability was assessed by MTT assay (12).

**Subcutaneous growth of human breast tumors in animals and treatments.** 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 ER-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); RIS at 150 μg/kg body weight; human PBMCs (3.5×10^7) injected intraperitoneally plus 10,000-U IL-2 administered alone or with 150-μg/kg RIS. In the relevant groups, IL-2 and RIS in 0.5-mL PBS were administered intraperitoneally every other days for 14 days. Tumor size was calculated by external measurement of the width (m_1) and length (m_2) of subcutaneous 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, anesthesized mice were culled by cervical dislocation and tumors collected for immunohistochemistry, real-time polymerase chain reaction (PCR) and IPP/ApppI phosphoantigen measurements.

Combination experiments, using a sequential treatment with doxorubicin followed by ZOL, were conducted as described by Ottewell et al. (16). Briefly, 5-week-old female SCID-NOD mice bearing subcutaneous B02 tumor xenografts were injected intraperitoneally on day 27 after tumor cell inoculation with (1) PBS, (2) doxorubicin (2mg/kg body weight), (3) doxorubicin followed 24 hours later by ZOL (100 μg/kg body weight), or (4) doxorubicin followed 24 hours later by ZOL+PBMC+IL2. Doxorubicin treatment was administered again
on day 33 after tumor cell inoculation. In the relevant groups, ZOL and IL-2 were administered intraperitoneally every other day for 14 days. Tumor size was measured as described above.

**IPP/ApppI Analysis.** Bisphosphonate-induced IPP/ApppI production was measured in human cancer cell lines *in vitro* and in subcutaneous tumors *in vivo*, as previously described (17). Briefly, following bisphosphonate treatments, tumor cells were scraped, washed in PBS and extracted using ice-cold acetonitrile (300 µL) and water (200 µL) containing 0.25-mM NaF and Na3VO4 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 and tumor extracts were then quantified by high-performance liquid chromatography negative ion electrospray ionization mass spectrometry.

**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. Vy9Vδ2 T cells and proliferating tumor cells were immunodetected using a FITC-conjugated anti-human TCR δ2 monoclonal antibody (Beckman Coulter) and a rabbit polyclonal anti–Ki-67 antibody (Abcam), respectively, as previously described (12).

**Real-time PCR.** Total RNA was extracted from T47D and B02 tumors and infiltrating Vy9Vδ2 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′caaggagctgggaagtgctgt; 3′cagctttttccacagatgctgc, human TCR Vδ2 (162 bp): 5′caaaaccatccgtttttgct; 3′cttgacagcattgatctcc, and ICAM-I (406bp): 5′ aggccaccccagaggaca; 3′cccattagtgcggctgctgta. 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.

**Flow-cyt fluorimetic analysis.** T47D, BO2 and MCF-7 breast cancer cell lines were seeded at a density of 1.10⁶ cells/well in 6-well plates. After overnight incubation the cells were incubated at 37°C for 24h with PBS, 10ng/ml TNF-α, 0.5ng/ml INF-γ (Biosource, Nivelles, Belgium) or conditioned medium from RIS-treated PBMCs. After this induction time, cells were stained with a phycoerythrin-conjugated human anti-ICAM-1 (CD54) (Beckman Coulter, Marseille, France) monoclonal antibody. All cells were re-suspended in 500µl PBS supplemented with 1% BSA and analyzed immediately by cytometry (Canto II, Becton Dickinson). Similar experimental conditions were conducted with untreated T47D, BO2 and MCF-7 cancer cells using phycoerythrin-conjugated monoclonal antibodies directed against anti-adhesion molecule receptors Alcam (CD166) (Beckman Coulter, Marseille, France), and MICA/B (eBioscience, San Diego, CA, USA).

**Statistical Analysis.** All data were analyzed using StatView software (version 5.0; 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

N-BPs induced human Vγ9Vδ2 T-cell expansion in vitro and in vivo.

The PBMCs were treated with different N-BPs in order to evaluate the relation between Vγ9Vδ2 T cell proliferation and inhibition of recombinant human FPPS in vitro. Only potent FPPS inhibitors such ZOL [half-maximal inhibitory concentration of FPPS activity (IC_{50}): 4.1 nM], RIS (IC_{50}: 5.7 nM) and, to a lower extent, the structural RIS analogue NE-58025 (IC_{50}: 42 nM) significantly stimulated γδ T-cell proliferation in vitro (Fig. 1A). By contrast, structural RIS analogues NE-58051 (IC_{50}: 337 nM) and NE-10790 (IC_{50}: 250×10^3 nM) had no effect (Fig. 1A). Thus, there was a strong correlation between FPPS inhibition and Vγ9Vδ2 T-cell expansion induced by N-BPs in vitro.

The treatment of human PBMCs with increasing concentrations of RIS stimulated in a time- and dose-dependent manner the expansion of Vγ9Vδ2 T cells (Supplementary Fig. S1A). Concomitant to Vγ9Vδ2 T cell expansion, mass spectrometry revealed rapid IPP/ApppI accumulation in PBMCs after RIS treatment (Supplementary Figs S1B and S1C). These results extend previous findings showing that ZOL stimulates Vγ9Vδ2-T cell proliferation and IPP/ApppI formation in PBMCs in vitro (12). This effect was specific to Vγ9Vδ2 T cells. Neither human γδ T cells expressing the Vγ9Vδ1 TCR, nor human αβ T cells, monocytes and B cells are responsive to N-BPs (13).

Murine PBMCs treated with N-BPs do not activate murine or human γδ T cells (7,13). The effect of RIS on Vγ9Vδ2-T cell expansion was therefore studied in vivo after intraperitoneal injection of human PBMCs into SCID-NOD immunodeficient mice and RIS+IL-2 treatment for 14 days. RIS (30, 150, 300, 600 μg/kg), in the presence of IL-2, dose-dependently stimulated the expansion of Vγ9Vδ2 T cells such that they comprised up to 50% of human T lymphocytes, versus < 10% for IL-2 alone or IL-2 plus RIS at a dose of 150 μg/kg (Fig. 1B). We previously observed similar results with the effect of ZOL treatment on Vγ9Vδ2-T-cell expansion from human PBMCs in vivo (12). This is explained by the fact that both ZOL and RIS are internalized by antigen-presenting cells from human PBMCs, leading
to intracellular IPP/ApppI accumulation and subsequent activation of Vy9Vδ2 T cells in a TCR-dependent manner.

**RIS induced IPP/ApppI production in human cancer cell lines in vitro**

We have previously shown that ZOL induces IPP/ApppI accumulation in human breast cancer cell lines (MCF-7, T47D, BT-474) having a luminal molecular subtype [ER-positive and/or progesterone receptor (PR)-positive and Ki-67-positive], whereas it does not in basal (ER-, PR- and HER2-negative) breast cancer cell lines (MDA-MB-231, MDA-MB-231/B02, MDA-MB-435, MDA-MB-435s, MCF10A) (12,17). Here, we report similar results with RIS (Supplementary Fig. 2A). RIS caused IPP/ApppI accumulation in 3 of the 4 luminal breast cancer cell lines studied (MCF-7, T47D, BT-474, ZR-75-1). By contrast, it did not induce IPP/ApppI formation in basal MDA-MB-231/B02 breast cancer cells.

IPP/ApppI accumulation in RIS-treated, ER-positive T47D cells was time-dependent, with IPP reaching a maximum concentration at 8 hours (721 pmol/mg protein) and ApppI peaking at 24 hours (426 pmol/mg protein) (Supplementary Fig. 2B). There was a time difference between maximum levels of IPP and ApppI, confirming that ApppI resulted from covalent binding of IPP and AMP (2). Because differences in the cellular uptake of RIS might also explain variation in IPP/ApppI production between breast cancer cell lines, the uptake of fluorescently labeled-RIS (FAM-RIS) was measured in T47D, MCF-7 and B02 cells. FAM-RIS uptake in T47D and MCF-7 cells was similar, but 2-fold lower in B02 cells (Supplementary Fig. 2C). We previously reported similar findings for the cellular uptake of ZOL (12). Additionally, we showed that HMG-CoA reductase expression, a mevalonate pathway enzyme upstream of FPP synthase, was low in B02 cells compared to that observed in T47D and MCF-7 cells (12). These results agree with an earlier report (18) that high HMG-CoAR expression correlated positively with ER expression in tumor tissue from patients with breast cancer. Taken together (12, 18, and this study) these results demonstrate that IPP/ApppI production in breast cancer cells depends on cellular uptake of NBP and on mevalonate pathway activity associated with the ER status of the cell.
RIS-induced IPP/Apppl accumulation in human breast cancer cell lines correlates with Vy9Vδ2 T-cell–mediated cancer cell death in vitro

We next examined whether the anticancer potency of Vy9Vδ2-T cells might depend on intracellular IPP/Apppl production in RIS–treated ER-positive and -negative breast cancer cells in vitro and in vivo. A 1-hour treatment with RIS (1–25 μM) did not affect survival of T47D, MCF-7, BT-474, ZR 75-1 and B02 cells in vitro (Figs. 2A, 2C, 2E, 2G and 2I, respectively). However, co-culturing RIS-treated T47D and MCF-7 cells with purified Vy9Vδ2T cells led to dose-dependent cancer cell death, which was statistically significant with RIS at concentrations as low as 3 μM for 1 hour (Figs. 2B and 2D). Similarly, Vy9Vδ2 T cells mediated cytotoxicity of RIS-treated BT-474 cells (Fig. 2F). A minimum concentration of 25 μM RIS was however required to trigger the cytotoxicity of Vy9Vδ2T cells against this breast cancer cell line. In sharp contrast, Vy9Vδ2 T cells did not exhibit cytotoxicity against RIS-treated ZR 75-1 and B02 cells (Fig. 2H and 2J). Similarly, coculture of ZOL-treated T47D and MCF-7 cells with purified Vy9Vδ2 T cells leads to dose-dependent cancer cell death, whereas Vy9Vδ2 T cells are not cytotoxic against ZOL-treated B02 cells (12). Altogether, these results strongly suggested that N-BP-induced IPP/Apppl accumulation in breast cancer cells was responsible for Vy9Vδ2-T cell-mediated cancer cell death. Further experiments were therefore conducted in vivo, using breast tumors cells with low or high IPP/Apppl levels after N-BP treatment.

Cytotoxic effect of Vy9Vδ2 T cells against human breast tumor xenografts in vivo

We observed that RIS+IL-2 treatment of SCID-NOD mice engrafted with human PBMCs stimulated the expansion of Vy9Vδ2 T cells in vivo (Fig. 1B). Vy9Vδ2 T cells are known only to exist in primates and humans (2,13), and they only recognize tumor cells of human origin (13), making our animal model most suitable for studying the potential role of RIS (and ZOL) in cancer immunotherapy. Of note, this effect of RIS on in-vivo Vy9Vδ2-T-cell expansion was observed at a dosing regimen calculated equivalent to 525 μg/kg body weight, weekly, whereas the approved weekly dose of RIS for prevention of aromatase
inhibitor-induced bone loss in breast cancer is 600 µg/kg body weight (14), further supporting the clinical relevance of our animal model. Therefore, *in vivo* experiments were conducted with SCID-NOD mice that were bearing subcutaneous T47D or B02 breast tumor xenografts. Treatment with RIS alone or PBMC+IL-2 did not inhibit T47D and B02 tumor growth (Figs 3A and 3B). By contrast, a 46% reduction of the volume of T47D tumors (*P*<0.05) was observed in mice receiving PBMC+IL-2+RIS, when compared to placebo (Fig. 3A). A similar treatment of B02 tumor-bearing animals with PBMC+IL2+RIS did not inhibit tumor growth (Fig. 3B).

In agreement with *in vivo* data, the *in situ* immunodetection of the Ki-67 nuclear antigen in T47D tumors from animals treated with PBMC+IL2+RIS showed a reduction in the proliferative index, when compared with that observed with tumors from placebo-treated animals (Fig. 3C). By contrast, the proliferative index of B02 tumors from animals treated with PBMC+IL2+RIS did not differ significantly from that obtained with placebo (Fig. 3D). Of note, Vy9Vδ2 T-cell infiltrates were detected in T47D but not B02 tumors from mice treated with PBMC+IL-2+RIS, as judged by RT-PCR (Figs. 3E and 3F).

**Vy9Vδ2 T-cell–mediated cancer cell death *in vivo* correlates with RIS-induced ApppI accumulation in breast tumor xenografts**

Because Vy9Vδ2 T cells were infiltrating subcutaneous T47D tumor xenografts in mice treated with PBMC+IL-2+RIS (Fig. 3E), mass spectrometry analysis of tumor extracts was performed in order to measure ApppI contents. ApppI was barely detectable in T47D tumors from placebo-treated mice (Fig. 4A), whereas substantial ApppI amounts were detected 48 hours after PBMC+IL-2+RIS treatment (Fig. 4B). ApppI was also detected in T47D tumor extracts from mice treated with RIS alone (data not shown). In contrast, RIS-induced ApppI production was not detected in B02 tumor xenografts (data not shown). Low IPP levels were detected at 48 hours after the last RIS dose in PBMC+IL-2+RIS-treated mice (not shown). We previously reported similar results regarding IPP/ApppI accumulation in T47D tumor extracts 48 hours after the last treatment of mice with PBMC+IL-2+ZOL (12). Conversely, IPP levels were high in T47D tumor extracts at 24 hours after the last ZOL dose,
whereas ApppI levels were barely detectable (12). We did not measure here IPP/ApppI levels in tumor extracts 24 hours after the last RIS dose in PBMC+IL-2+RIS-treated animals. Nevertheless, our findings (12 and this study) are consistent with the fact ApppI formation results from the intracellular accumulation of IPP after FPPS inhibition (2). Importantly, as shown here (Figs. 3 and 4) and in our previous study (12), N-BP uptake in tumors (as judged by IPP/ApppI production) is not enough for tumor growth reduction in vivo. Indeed, tumor growth reduction coincides with infiltration of Vγ9Vδ2 T cells in tumors that produced IPP/ApppI.

**Induction of ICAM-1 expression in breast cancer cells *in vitro* and *in vivo***

Vγ9Vδ2 T cells recognize N-BP-treated tumor cells through co-stimulatory non-TCR receptors such as CD6, NKG2D and LFA1 in vitro (13). However, there is no evidence of their engagement for recognition of N-BP-treated tumors by Vγ9Vδ2 T cells in vivo. The presence of ligands for these cell surface receptors was evaluated by flow cytometry using T47D, MCF-7, and B02 breast cancer cell lines (Supplementary Fig. 3). Cell adhesion molecule Alcam (CD166), the ligand for CD6, was strongly expressed in all 3 cell lines, whereas MICA/B (NKG2D ligand) was not expressed. ICAM-1 (CD54), the ligand for LFA-1, was poorly expressed by all 3 cell lines. ICAM-1 is a member of the transmembrane immunoglobulin superfamily whose expression is induced by TNF-α or IFN-γ (19). Additionally, RIS and ZOL both strongly induced IFN-γ production by PBMCs in vitro (data not shown). We therefore performed experiments with the conditioned medium from RIS- and ZOL-treated PBMCs to induce cell adhesion molecule expression in breast cancer cells. ICAM-1 expression was significantly increased by conditioned media from N-BP-stimulated PBMCs in all 3 cell lines, when compared with IFN-γ or TNF-α (Fig. 5A). MICA/B was not expressed under these experimental conditions (data not shown).

We next examined whether ICAM-1 could be induced in experimental human breast tumors *in vivo*. In our animal models of breast cancer, RT-PCR showed a 3-fold increase ICAM-1 expression in T47D tumors from mice treated with PBMC+IL-2+RIS or PBMC+IL-
2+ZOL versus placebo (Figs. 5B and 5C). T47D tumors from mice treated with RIS (or ZOL) alone or PBMC+IL-2 showed a modest but significant increased expression of ICAM-1 versus placebo (Figs. 5B and 5C). In sharp contrast, ICAM-1 was poorly expressed in B02 tumors, irrespective of the presence or absence of PBMCs and/or the use of a N-BP (RIS or ZOL) (Figs. 5B and 5C). These results may be explained by the fact that tumor-derived IPP/Apppl phosphoantigens behave like rate-limiting factors. We have previously shown that phosphoantigen production by tumor cells recruits Vγ9Vδ2T cells to breast tumors in vivo (12). Infiltrating Vγ9Vδ2T cells then secrete IFN-γ that is growth inhibitory for tumor cells (7,12,13). Here, because of the lack of Apppl production by B02 cells, Vγ9Vδ2T cells did not infiltrate B02 tumor xenografts (Fig. 3F). Consequently, there was no production of IFN-γ by Vγ9Vδ2T cells and no activation of ICAM-1 expression within B02 tumors. This contention was supported by serum measurements of human IFN-γ. We observed that IFN-γ levels were high-to-moderate in T47D-tumor-bearing mice treated with PBMCs+IL2+RIS (765 ± 357 pg/ml, n=6) or PBMCs+IL2 (563 ± 370, n=5), whereas the cytokine was barely detected in B02-tumor bearing animals treated with PBMCs+IL2+RIS (22 ± 45 pg/ml, n=5) or PBMCs+IL2 (12 ± 23 pg/ml, n=5) (P < 0.001).

Thus, our study establishes that IPP/Apppl-producing tumors induce Vγ9Vδ2T-cell infiltration and secretion of IFN-γ which, in turn, triggers the recognition of tumors by Vγ9Vδ2T cells through ICAM-1 engagement, leading to tumor growth reduction in vivo.

**A sequential treatment with doxorubicin and ZOL sensitizes unresponsive B02 breast tumors to Vγ9Vδ2T-cell cytotoxicity in vivo.**

We next determined whether combination of a N-BP treatment with chemotherapy might sensitize low IPP/Apppl-producing B02 tumors to Vγ9Vδ2T-cell cytotoxicity in vivo. It has been previously shown by Ottewell et al. (16) that a sequential treatment with doxorubicin followed 24 hours later by ZOL reduces subcutaneous growth of ER-negative MDA-MB-436 tumors in animals. Additionally, Mattarollo et al. (20) have shown that
chemotherapy and ZOL sensitize ER-negative MDA-MB-231 breast cancer cells to Vγ9Vδ2T-cell cytotoxicity in vitro. The human B02 breast cancer cell line used in our study is a subpopulation of MDA-MB-231 (15). Since B02 cells were not susceptible to ZOL-mediated Vγ9Vδ2T-cell cytotoxicity, we therefore performed combination experiments using a sequential treatment with doxorubicin followed by ZOL, alone or in combination with PBMC+IL2 (Fig. 6). Doses of doxorubicin (2 mg/kg body weight) and ZOL (100 μg/kg body weight) given to animals were calculated clinically equivalent to doses given in patients (16).

On the first week of treatment, tumor-bearing animals treated with doxorubicin followed by ZOL+PBMC+IL2 had statistically significantly smaller tumors (115 ± 43 mm³) than those of animals treated with doxorubicin+ZOL (228 ± 54 mm³; P=0.0007) or with doxorubicin alone (242 ± 72 mm³; P=0.0004) (Fig. 6). However, on the second week of treatment, tumors from animals treated with doxorubicin followed by ZOL+PBMC+IL2 reached volumes (254 ± 94 mm³) that were similar to tumor volumes of animals treated with doxorubicin+ZOL (300 ± 44 mm³; P=0.47), whereas there was still a modest inhibitory effect compared with animals treated with doxorubicin alone (390 ± 143 mm³; P=0.05) (Fig. 6). Ottewell et al. (16) have previously shown that a 6-week course of sequential administration of doxorubicin followed by ZOL elicited antitumor effects in animals. Here, we only used a 2-week course of sequential treatment, which probably explained why doxorubicin followed by ZOL did not enhance the antitumor effect of the chemotherapy. However, the engraftment of PBMC+IL2 to animals enhanced the antitumor effect of this sequential treatment by promoting the expansion and cytotoxicity of Vγ9Vδ2T cells against B02 cells. These results were in line with previous findings (20) showing that chemotherapy and ZOL sensitize solid tumor cells to Vγ9Vδ2T-cell cytotoxicity in vitro. The antitumor effect observed here was however transient. It may be explained by IPP/AppI levels in B02 tumors which were probably too low to efficiently recruit Vγ9Vδ2T cells to the tumor site. In this respect, AppI measurements in B02 tumor extracts 48 hours after the last ZOL dose in mice treated with doxorubicin followed by ZOL+PBMC+IL2 showed that phosphoantigen amounts did not differ significantly from that
measured in tumors from animals treated with doxorubicin followed by ZOL (120 ± 85 versus 113 ± 80 nM). Low ApppI amounts were detected in tumor extracts from animals treated with the placebo (45 ± 35 nM) or doxorubicin alone (75 ± 65 nM). Nevertheless, our findings showed that a sequential treatment with doxorubicin followed by ZOL+PBMC+IL-2 increased cytotoxic activity of Vγ9Vδ2 T cells against low IPP/ApppI-producing breast tumors in vivo.

**Conclusion**

Our study provides new insights into how N-BP (RIS and ZOL) could empower the immune system to pinpoint breast tumors and inhibit their outgrowth. We showed that ER-positive breast tumors were more likely to produce phosphoantigens IPP/ApppI after N-BP treatment compared with ER-negative breast tumors, thereby promoting Vγ9Vδ2 T-cell activation in vivo. Moreover, the ability of RIS and ZOL to activate Vγ9Vδ2T cells not only depended on IPP/ApppI accumulation in tumors but also on expression of tumor cell surface receptor ICAM-1, which triggered the recognition and killing of bisphosphonate-treated breast cancer cells by Vγ9Vδ2T cells.

There is clinical evidence that ZOL durably activates γδT cells in patients with early breast cancer (21). In other small trials, the modulation of γδT cells with ZOL has been shown to improve clinical outcome in patients with lung or prostate cancer (22). In large phase-III clinical trials, it has been shown that adding ZOL to standard systemic therapy (endocrine therapy or chemotherapy) improves disease-free survival and overall survival of patients with ER-positive breast cancer in a low estrogen environment (i.e., following ovarian suppression therapy or in women with established menopause at diagnosis) (4-6). The mechanisms responsible for this antitumor effect of ZOL in early breast cancer are unknown but may involved changes in the bone marrow environment and on disseminated tumor cells, which can prevent metastatic disease and local recurrences (4,5,22). Our present present findings suggest that the immunomodulating role of NBPs on human γδT cells may have also contributed to the anticancer effects of ZOL observed in these clinical trials. Therefore, we
conclude that adjuvant therapy with NBP might be an effective strategy to activate antitumor immunity in patients with ER-positive breast cancer.

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REFERENCES


FIGURE LEGENDS

Figure 1. Correlation between inhibition of recombinant human FPP synthase by N-BPs in vitro and expansion of human Vγ9Vδ2 T cells induced by N-BPs in vitro and in vivo. (A) PBMCs were treated with 10μM ZOL, RIS, NE-58025, NE-58051, or NE-10790 plus 100U/mL IL-2, or with IL-2 alone. Vγ9Vδ2T cells proliferation was analyzed by flow cytometry after 14 days culture. Data are expressed as mean ± SD with PBMCs from three independent blood donors. Half-maximal inhibitory concentrations (IC50) for in-vitro inhibition of recombinant FPP synthase are in the nM range and were obtained from Stresing et al. (3). (B) Effect of RIS on Vγ9Vδ2T-cell expansion from human PBMCs in vivo. Proportion of human Vγ9Vδ2T cells determined by flow cytometry from peritoneal cells of immunodeficient mice receiving a single intraperitoneal injection of human PBMCs and 10, 30, 150, 300, or 600 μg/kg RIS plus 10,000 U IL-2 or IL-2 alone every 2 days for 14 days. Results are mean±SD of 5 mice per group. **p<0.01. Abbreviations: PBMCs, peripheral blood mononuclear cells; FPPS, farnesyl pyrophosphate synthase; RIS, risedronate; ZOL, zoledronic acid.

Figure 2. RIS-induced IPP/ApppI phosphoantigen accumulation in human breast cancer cells correlates with Vγ9Vδ2Tcell–mediated cancer cell death in vitro. T47D, MCF-7, BT-474, ZR 75-1 and B02 cells were pretreated with control (CTR; PBS) or 1–25 μM RIS for 1h, then cultured without drug for 18h. After incubation, cells were cocultured without (A, C, E, G, I) or with (B, D, F, H, J) purified Vγ9Vδ2 T cells for 24h. The ratio of cancer cells to Vγ9Vδ2 T cells was 1:12.5. (A–J) are representative of 3 independent experiments (mean±SD). *P<.05; **P<.01. Abbreviations: Apppl, triphosphoric acid I-adenosin-5’-yl ester 3-(3-methylbut-3-enyl) ester; IPP, isopentenyl pyrophosphate; RIS, risedronate.

Figure 3. Cytotoxic effect of Vγ9Vδ2 T cells against human T47D and B02 breast 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 PBMCs from
healthy human adult blood donors. Additionally, mice were treated with IL-2±RIS (150 μg/kg) every 2 days for 2 weeks. (A, B) T47D and B02 tumor growth was determined at day 26, day 34 and day 42 after tumor cell inoculation, by measurement of the tumor volume with a Vernier caliper. (C, D) T47D and B02 Ki-67 proliferative indices were assessed by immunohistochemistry. (E, F) Relative expression of Vγ9Vδ2 T cells in T47D and B02 tumors was assessed by RT-PCR. Results are the mean±SD of 6–7 mice per group. ns, not significant; *P<.05 and **P<.01. Abbreviations: IL-2, interleukin-2; PBMCs, peripheral blood mononuclear cells; RIS, risedronate.

Figure 4. Mass spectrometry analysis of phosphoantigen ApppI in T47D breast tumor xenografts. Identification of ApppI in T47D tumors of placebo- and RIS-treated mice was performed by high-performance liquid chromatography negative ion electrospray ionization mass spectrometry. Chromatograms correspond to tumor extracts obtained from (A) placebo-treated mice and (B) animals that received PBMC+IL-2+RIS whose tumors were collected 48h after the last RIS injection. Similar chromatograms were obtained with tumor extracts from mice treated with RIS alone. Tumor extracts were spiked with 0.11-pmol ApppI. ApppI chromatograms are drawn on the same scale. Results are mean±SD of 6–7 mice per group. ns, not significant (P>.05); *P<.05. Abbreviations: ApppI, triphosphoric acid I'-yl ester 3-(3-methylbut-3-enyl) ester; IL-2, interleukin-2; PBMC, peripheral blood mononuclear cells.

Figure 5. Induction of ICAM-1 expression on T47D, MCF-7, and B02 breast cancer cells by cytokines released from Vγ9Vδ2 T cells in vitro and in vivo. (A) T47D, MCF-7, and B02 breast cancer cells were left untreated or treated with 10 ng/mL TNF-α, 0.5 ng/mL IFN-γ, or condition medium from RIS-stimulated PBMC after 4 days. Percentage expression of ICAM-1 was determined by flow-cytometric analysis. (B) Mice were inoculated subcutaneously with T47D or B02 cells. Four weeks after tumor cell inoculation, when tumors became palpable, mice were engrafted with PBMCs from healthy human adult blood donors.
Additionally, mice were treated with IL-2±RIS (150 μg/kg) every 2 days for 2 weeks. Relative ICAM-1 expression in T47D and B02 tumors from mice treated with RIS was determined by RT-PCR. (C) same as (B) using IL-2±ZOL (30 μg/kg) every 2 days for 2 weeks. Results are mean±SD of 6–7 mice per group. ns, not significant (P > .05); *P < .05; **P < .01; ***P < .001.

Abbreviations: CM, conditioned medium; IL-2, interleukin-2; PBMC, peripheral blood mononuclear cells; RIS, risedronate; ZOL, zoledronate.

Figure 6. Effect of doxorubicin administered in sequence with zoledronate, alone or in combination with PBMC+IL2, on growth of B02 breast tumors. Mice were inoculated subcutaneously with B02 cells. On day 27 after tumor cell inoculation, when tumors became palpable, mice were injected once per week for 2 weeks with 2 mg/kg doxorubicin or saline. Mice receiving doxorubicin were randomized into 3 treatment groups. The first group of animals received doxorubicin alone. The second group of animals received doxorubicin followed 24 hours later by 100 μg/kg ZOL. Animals from the third group that received doxorubicin were engrafted 24 hours later with PBMCs from healthy human adult blood donors and were treated with ZOL+IL-2. In the relevant groups, IL-2±ZOL (100 μg/kg) was then administered every other days for 2 weeks. Tumor growth was determined at day 26, day 34 and day 42 after tumor cell inoculation, by measurement of the tumor volume with a Vernier caliper. Results are the mean±SD of 6–7 mice per group. *P < .05 and **P < .01.

Abbreviations: IL-2, interleukin-2; PBMCs, peripheral blood mononuclear cells; DOX, doxorubicin; ZOL, zoledronate.
Figure 1

A

Proportion of Vγ9Vδ2 T cells (%)

IC₅₀ to inhibit FPPS (nM)

ZOL
RIS
NE-58025
NE-58051
NE-10790

B

Proportion of Vγ9Vδ2 T cells, %

0 10 30 150 300 600

RIS (µg/kg)
Figure 2

IPP/Apppl levels

High

T47D

MCF-7

Medium

BT-474

Low

ZR 75-1

B02
Figure 4

**Standard**

**Tumor cells** → **Human PBMCs** → **MS**

**A** Placebo

**B** RIS, 48 h

**T47D Tumors**

- Placebo
- RIS
- PBMC + IL2
- PBMC + IL2 + RIS

Tumor volume (mm³) after 2-week treatment

- Placebo
- RIS
- PBMC + IL2
- PBMC + IL2 + RIS

ns

*
Figure 5

A

<table>
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<th></th>
<th>Untreated</th>
<th>TNF-α (10ng/ml)</th>
<th>INF-γ (0.5ng/ml)</th>
<th>CM from RIS-treated PBMCs</th>
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<td>60.4%</td>
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<tr>
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<td>84.1%</td>
<td>90.7%</td>
<td>96.9%</td>
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</table>

ICAM-1 Expression

B

RIS: Risedronate

C

ZOL: Zoledronate

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Figure 6

![Graph showing tumor growth and treatment timeline.](image-url)

- **Tumor Volume**
- **D0** to **D40** timeline with treatments indicated:
  - **D0**: Tumor injection (B02 Tumor)
  - **D27**: Doxorubicin injection
  - **D33**: ZOL injection ± IL2
  - **D40**: Tumor volume measurement

**PBMC** injection at **D40**.

**Graph** showing tumor volume over time:

- **Placebo**
- **DOX**
- **DOX + ZOL**
- **DOX + ZOL + PBMC + IL2**

Tumor volume in mm³ plotted against days from 25 to 45.
In-Vivo Phosphoantigen Levels in Bisphosphonate-Treated Human Breast Tumors Trigger Vδ9Vγ2 T-Cell Antitumor Cytotoxicity through ICAM-1 Engagement.

Ismahene Benzaïd, Hannu Mönkkönen, Edith Bonnelye, et al.

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