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Zoledronate Enhances the Cytotoxicity of Gamma Delta T Cell Immunotherapy in an Orthotopic Mouse Model of Osteolytic Osteosarcoma

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Abstract

Objective: Osteosarcoma is the most common primary tumor of the bone, predominantly affecting children and adolescents. While localized osteosarcoma can be readily treated with the use of pre-operative chemotherapy in combination with surgery, patients who develop metastatic disease and tumor-induced osteolysis continue to have a poor prognosis. Many cancer cells express tumor-specific antigens, rendering them vulnerable to immune effector T cell killing. There is increasing evidence that highly cytotoxic gamma delta (Vγ9Vδ2) T cells together with the bone anti-resorptive drug zoledronate may hold significant clinical benefit in the treatment of a variety of tumor types.

Methods: *Ex vivo* expanded Vγ9Vδ2 T cells were used to assess effector-mediated killing of osteosarcoma cells (BTK-143 and K-HOS) in response to zoledronate pre-treatment. An orthotopic mouse model of osteolytic osteosarcoma was used to verify Vγ9Vδ2 T cell cytotoxicity in combination with zoledronate on tumor growth, osteolysis and metastasis.

Results: Pre-treatment of osteosarcoma cells with zoledronate enhanced Vγ9Vδ2 T cell rapid killing compared to untreated cells *in vitro via* blockade of the mevalonate pathway. When adoptively transferred into osteosarcoma bearing NOD/SCID mice *in vivo*, Vγ9Vδ2 T cells in combination with zoledronate potentiated the anti-cancer efficacy of Vγ9Vδ2T cells and inhibited tumor induced osteolysis. Importantly, Vγ9Vδ2 T cells alone reduced both the incidence and burden of lung metastases.

Conclusion: This study demonstrated the dual-action of zoledronate to enhance the immunogenicity of osteosarcoma cells to Vγ9Vδ2 T cell cytotoxicity and provide protection against tumor-induced osteolysis.

Keywords: Gamma delta T cell; Immunotherapy; Osteolysis; Osteosarcoma; Zoledronate

Abbreviations: E:T- Effector Target Ratio; FBS- Foetal Bovine Serum; IL-2- Interleukin-2; IPP- Isopentyl Pyrophosphate; i.t- Intratibial; i.v- Intravenous; LDH- Lactate Dehydrongenase; MHC- Major Histocompatibility Complex; NOD/SCID- Non-Obese Diabetic/ Severe Combined Immunodeficiency; PBMC- Peripheral Blood Mononuclear Cells; PBS- Phosphate Buffered Saline; PVDF- Polyvinylidene Difluoride; RAP1- Ras-Related Protein 1; TBST- Tris Buffered Saline-Tween 20; T.BV- Total Bone Volume; Tr.BV- Trabecular Bone Volume; Vγ9Vδ2 T cells- Vgamma9 Vdelta2 T cell; ZOL- Zoledronate

Introduction

Osteosarcoma is the most frequent primary malignancy of the bone occurring in children and adolescents [1]. Advances in current treatments, including neo-adjuvant chemotherapy followed by tumor resection, have resulted in a 10-year overall survival rate of 70-80% for patients with localized osteosarcoma [2]. However, off-target toxicity and the emergence of drug resistance can limit treatment efficacy and severely impact patient's quality of life. Aggressive osteosarcomas are characterized by a high rate of tumor growth, tumor-induced osteolysis and preferential metastases to the lungs, leading to poor patient survival [3-6].

Immunotherapy has been reported to hold substantial promise to improve the outcomes of patients with osteosarcoma [7]. To this end, adoptive transfer of gamma delta (γδ) T cells has gained momentum as a potential new immunotherapeutic approach for targeting various

solid and hematological malignancies [8-11]. The majority of γδ T cells in human peripheral blood express Vγ9Vδ2 T cell receptors and constitute 1-10% of circulating lymphocytes [12]. These unique T cells can kill a broad range of tumors in a MHC-unrestricted manner, as well as producing high levels of anti-tumor cytokines and cytotoxic granules [13]. It is well documented that Vγ9Vδ2 T cells can be activated and expanded by zoledronate (ZOL), a third-generation nitrogen-containing bisphosphonate already used in cancer patients with osteosarcoma and skeletal malignancies [14-17]. ZOL inhibits a key enzyme in the mevalonate pathway, leading to the accumulation of phospho-antigens, such as isopentenyl pyrophosphate (IPP) that is recognized by Vγ9Vδ2 T cells [18]. Notably, ZOL pre-treatment has been demonstrated to enhance the immunogenicity of cancer cells *via* the accumulation of IPP's, leading to their rapid killing by Vγ9Vδ2 T cells [17,19-21].

In the present study, we demonstrated that ZOL pre-treatment sensitized highly metastatic and osteolytic osteosarcoma cells to Vγ9Vδ2 T cell cytotoxicity *in vitro*. Furthermore, we showed that ZOL pre-treatment of osteosarcoma-bearing mice, significantly enhanced the anti-cancer efficacy of Vγ9Vδ2 T cells, greatly inhibited tumor-associated bone destruction *in vivo*, and reduced both the incidence and metastatic tumor burden within the lung. Thus, our study shows that adoptive transfer of Vγ9Vδ2 T cells in combination with ZOL has great potential as a novel immunotherapeutic approach for the treatment of osteolytic osteosarcoma.

Materials and Methods

Cells and reagents

BTK-143 and K-HOS human osteosarcoma cancer cell lines were obtained from ATCC (Manassas, VA, USA). Both cell lines express luciferase produced by retroviral expression of the SFG-NES-TGL vector [22]. Cancer cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM, Life Technologies, Australia) supplemented with 10% foetal bovine serum (FBS, Life Technologies, Australia), 100 IU/mL penicillin (Life Technologies, Australia), 100 μg/mL streptomycin (Life Technologies, Australia) and 25 mM HEPES (Life Technologies, Australia) at 37°C in a 5% CO_2 humidified atmosphere. ZOL was sourced from Novartis Pharma AG. Antibodies against RAP1 and RAP1A were purchased from Santa Cruz Biotechnology, USA.

Ex vivo expansion and enrichment of Vγ9Vδ2 T cells

Peripheral blood from healthy adult donors was obtained following informed consent and Vγ9Vδ2 T cells were expanded as previously described [23]. Briefly, peripheral blood mononuclear cells (PBMC) were isolated by density gradient centrifugation using Lymphoprep™ (Axis Shield, Norway). PBMC were cultured in complete CTS™ OpTmizer™ Medium supplemented with OpTmizer™ T cell Expansion Supplement (Life Technologies, Australia), at a density of 1×10^6 cells/mL in the presence of 5 μM ZOL and recombinant human IL-2 (100 IU/ml) (BD Pharmingen, USA) on the first day of culture, then supplemented with IL-2 every 3 days for the duration of the culture. Following 8-10 days of culture, cells were enriched using magnetic activated cell sorting (MACS) system using negative selection with the TCR γ/δ+ T cell Isolation Kit (human) (Miltenyi Biotec, Germany). Purity of Vγ9Vδ2 T cells was determined by flow cytometry analysis. The percentage of Vγ9Vδ2 T cells from donors was consistently >95%.

Cell cytotoxicity assay

The cytotoxicity of human *ex vivo* expanded Vγ9Vδ2 T cells against osteosarcoma cell lines was assessed using the lactate dehydrogenase (LDH) release assay (CytoTox 96® Non-Radioactive Cytotoxicity Assay; Promega, USA) as per the manufacturer's instructions. Briefly, 1×10^4 target cells (K-HOS or BTK-143) were seeded in a 96-well microtiter plate. After 24 hr, cells were pre-treated with or without 25 μM ZOL for a further 24 hr before being cultured with Vγ9Vδ2 T cells at various E: T (Effector: Target) ratios. After co-culture for 9 hr at 37°C, 50 μL of supernatant was removed and assayed for LDH activity. Absorbance (490 nm) was measured using a FLUOstar Optima plate reader (BMG Labtech) and LDH activity quantified.

Western blotting

Whole-cell lysates of BTK-143 and K-HOS cells were collected using RIPA lysis buffer (Sigma–Aldrich) supplemented with protease cocktail inhibitor (Roche, Basel, Switzerland) and phosphatase inhibitors (1 mM sodium vanadate and 0.5 mM sodium fluoride). Lysates were resolved on 4–12% Bis-Tris gels and electrophoretically transferred to Hybond-P PVDF membranes (GE Healthcare, UK). Membranes were blocked in 5% skim milk/TBST and incubated with 1/1000 dilution of primary antibody in 1% skim milk/TBST, followed by a 1/5000 dilution of alkaline phosphatase-conjugated secondary antibodies (Thermo Fisher Scientific, USA). Immobilized antibodies were detected with the ECF substrate reagent kit (GE Healthcare, UK) according to the manufacturer's instructions and antibody binding visualized using the LAS4000 imaging system (GE Healthcare). Protein levels were quantified by densitometric analysis using ImageJ 2014 (Version 1.49n, Bethesda, MD).

Animals

Animal studies were performed in accordance with the animal protocol procedures approved by the Animal Ethics Committee of The University of Adelaide and SA Pathology, and conform to the guidelines established by the 'Australian Code of Practice for the Care and Use of Animals for Scientific Purposes'.

In vivo anti-tumor efficacy of ZOL and Vγ9Vδ2 T cells

Orthotopic intratibial (i.t) injections were performed as previously described [22,24]. Briefly, five-week old female NOD/SCID mice were anaesthetized and a 27-guage needle coupled to a Hamilton syringe was used to inject 1×10^5 BTK-143 osteosarcoma cells in 10 μL of PBS, directly through the tibial plateau into the bone marrow space. The contralateral tibia served as the non-tumor bearing control. Once tumors were established, mice were assigned into four treatment groups (5 mice/group): Control (untreated), ZOL alone (100 μg/kg s.c), Vγ9Vδ2 T cells alone (1×10^7/100 μL injected *via* the tail vein), and ZOL+Vγ9Vδ2 T cells (infusion of Vγ9Vδ2 T cells 24 hr after ZOL). Treatments were given at Day 8 and 14. Tumor bioluminescence was monitored using the IVIS Spectrum as previously described [23]. After 3 weeks, mice were sacrificed, and lungs were imaged for *ex vivo* bioluminescence to quantify lung metastases. Tumor bearing and non-tumor bearing control tibia from each animal were surgically resected for micro-computed tomography.

Ex vivo micro-computed tomography (μCT) analysis

Mouse tibias for μCT analysis were scanned using the SkyScan-1076 high-resolution μCT Scanner (Bruker) as previously described [22,23]. Briefly, cross-sections were reconstructed using NRecon (V1.6.9.8, Bruker). Images were realigned in DataViewer (1.5.1.2, Bruker) and imported into CT Analyser (CTAn) (V1.14.4.1+, Bruker, Skyscan). Using the two-dimensional images obtained from the CTAn, the total bone (TBV) and trabecular (TbV) morphometric parameters were quantified. Representative three-dimensional images of total bone were generated in CTvox (V2.7.0, Bruker).

Statistical analysis

Data points derived from experiments are reported as the mean ± SEM. The statistical differences were detected by Student's t-test, and two-way ANOVA followed by multiple comparison test using the Bonferroni's method where indicated, using Sigma Plot 2011 12.5 (Systat Software Inc., USA). $p<0.05$ was considered statistically significant.

Results

ZOL pre-treatment enhances Vγ9Vδ2 T cell cytotoxicity against human osteosarcoma cell lines *in vitro* and is associated with inhibition of mevalonate pathway

The *in vitro* cytotoxicity of Vγ9Vδ2 T cells, in combination with ZOL, was evaluated against osteolytic BTK-143 and osteosclerotic K-HOS osteosarcoma cell lines. While Vγ9Vδ2 T alone exerted minimal cytotoxicity, ZOL pre-treatment of cancer cells resulted in a significant increase in cytotoxicity in both cell lines in an E: T dependent manner, from 25.6% to 53.4% for BTK-143 and from 15% to 39% for K-HOS cells when compared to untreated Vγ9Vδ2 T cell control (Figure 1A).

The increased cytotoxicity was associated with ZOL-mediated inhibition of prenylation, as evidenced by the accumulation of unprenylated Rap1A protein. As shown in Figure 1B and C, the accumulation of unprenylated Rap1 was evident after just 4 hr of exposure to ZOL (Figure 1B and C).

Adoptive transfer of Vγ9Vδ2 T cells in combination with ZOL reduces osteosarcoma burden in bone and in the lungs and protects against osteosarcoma-induced bone destruction

BTK-143 cells were directly inoculated into the left tibiae of NOD/SCID mice. Once tumors were established, ZOL was administered 24 hrs prior to the Vγ9Vδ2 T cell infusion at day 8 and 14. After 19 days, no difference in tumor burden between the untreated, ZOL alone, or Vγ9Vδ2 T cell alone treated groups was observed. In contrast, combination of ZOL and Vγ9Vδ2 T cell significantly decreased tumor growth (Figure 2A and B).

BTK-143 osteosarcoma cell growth within bone leads to the formation of predominantly osteolytic lesions [22]. Therefore, to evaluate the effects of Vγ9Vδ2 T cells alone or in combination with ZOL, on bone destruction, tibias were analyzed using three-dimensional (3D) μCT imaging (Figure 2C).

Untreated animals showed the greatest osteolysis with 19.2% total bone volume (T.BV) loss (Figure 2D) and a 43.6% loss of trabecular bone volume (Tb.BV) (Figure 2E). In contrast and in line with the well-known bone-protective effects of ZOL, treatment with ZOL alone limited the loss of bone, resulting in only 7.3% T.BV loss and 36.2% Tb.BV loss.

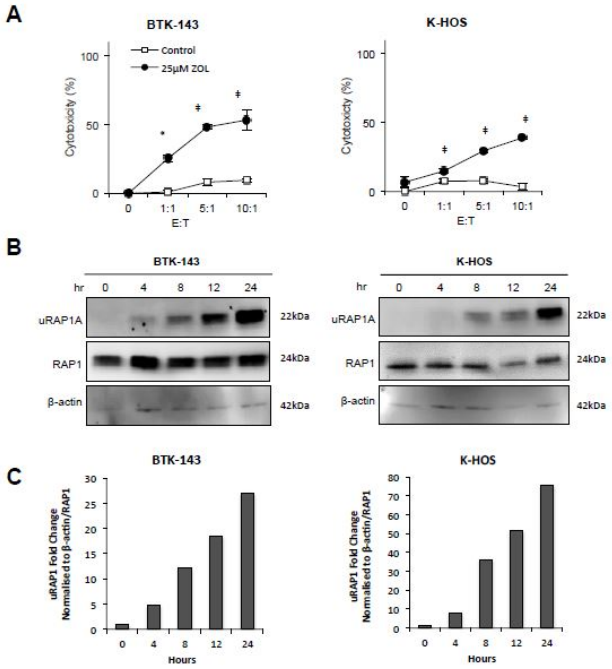

Figure 1: ZOL sensitizes osteosarcoma cells to Vγ9Vδ2 T cell cytotoxicity *in vitro*. Luciferase expressing BTK-143 and K-HOS osteosarcoma cells were pre-treated with culture media (Control) or ZOL at 25 μM for 24 hrs. Cancer cells were then co-cultured with an increasing E:T ratio of *ex vivo* expanded Vγ9Vδ2 T cells (1:1, 5:1, 10:1) for 9 or 24 hrs. **(A)** Vγ9Vδ2 T cell cytotoxicity was measured by LDH release following 9 hrs co-culture and indicated as percentage cell death of tumor targets. **(B)** BTK-143 and K-HOS osteosarcoma cells were treated with ZOL at 25 μM over 24 hrs at the designated times. Cell lysates were prepared in sampling buffer (1 × RIPA buffer, 10 mM sodium fluoride, 10 mM sodium vanadate) and then subject to Western immunoblot analysis with antibodies against total and unprenylated forms of RAP1. Antibody against β-actin served as loading control. Quantification of unprenylated RAP1A (uRAP1A) **(C)** Were normalized to β -actin/RAP1A using ImageJ software, and expressed as fold change compared to unstimulated control (0 hrs). Results are shown as the mean ± SEM of triplicate determinations performed in duplicate. Statistical significance was calculated by unpaired two-tailed Student's t-test (‡$p<0.0001$, †$p<0.001$, *$p<0.05$).

Animals treated with Vγ9Vδ2 T cells alone showed similar T.BV loss compared with untreated animals (23.7% compared to 19.2%), but greater Tb.BV loss (69.0% compared with 43.6%). In contrast, animals treated with ZOL in combination with Vγ9Vδ2 T cells, displayed a 1.5% gain in T.BV and only 8.4% Tb.BV loss.

To examine the efficacy of Vγ9Vδ2 T cells alone and in combination with ZOL on the incidence and tumor burden of lung metastases, tumor bioluminescence in the lungs was assessed (Figure 2F).

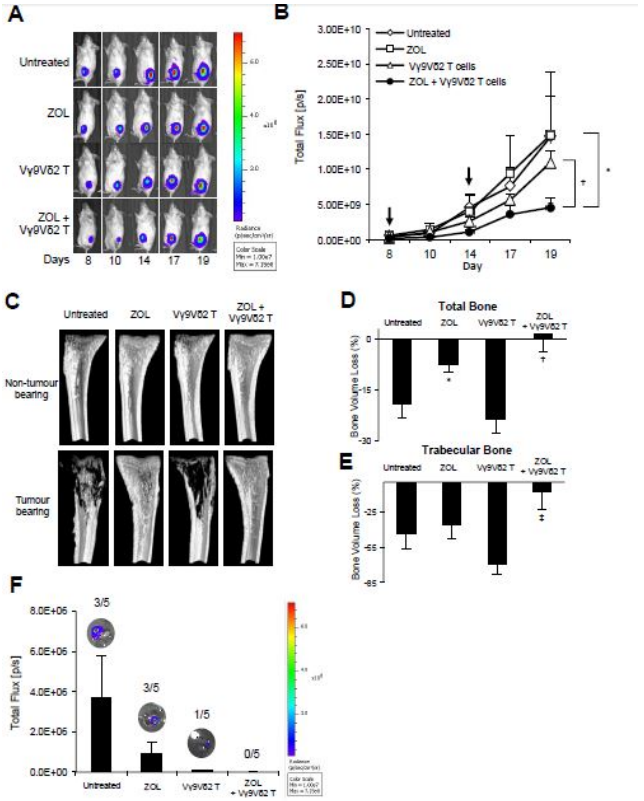

Figure 2: ZOL potentiates the anti-cancer efficacy of Vγ9Vδ2 T cells against osteolytic osteosarcoma. 5-week old female NOD/SCID mice were injected orthotopically with 1×10^5 BTK-143 cells into the left tibial cavity. Once tumors were established, treatments (ZOL 100 µg/kg s.c; Vγ9Vδ2 T cells 1×10^7/100 µL i.v) were commenced from Day 8. Mice were imaged weekly using the Xenogen IVIS-Spectrum bioluminescence imaging system. **(A)** Representative whole body BLI images of a single animal from each group (n=5) during the course of the experiment. **(B)** Average tumor signal over time expressed as mean Total Flux measured in photon counts per second (p/s) and administered treatments indicated by the arrows. **(C)** Representative µCT 3D images showing the osteolytic nature of BTK-143 osteosarcoma lesions in each group. Quantitative image analysis of **(D)** total bone and **(E)** trabecular bone volume is expressed as a percentage difference between the tumor bearing tibia and non-tumor bearing tibia. **(F)** Quantification of bioluminescence within the lungs, expressed as mean Total Flux, photon counts per second (p/s), with representative images of the lungs from each treatment group after 19 days. Results are shown as the mean ± SEM. Statistical significance was calculated by two-way ANOVA followed by multiple comparison test using the Bonferroni's method ($^{\dagger}p<0.001$, $^{*}p<0.05$).

Three out of the five mice in the untreated group exhibited lung metastasis, which was comparable to ZOL treatment alone, however, ZOL reduced tumor burden by more than half compared to untreated mice. Vγ9Vδ2 T cells alone and in combination with ZOL, reduced tumor incidence to one out of five and none of the animals in the combination treatment displayed lung metastases.

Discussion

Although osteosarcoma is considered an osteoblastic malignancy, aggressive osteosarcomas are associated with extensive osteolytic bone destruction. As a result, patients endure immense pain and are susceptible to pathologic bone fractures. Immunotherapy, using Vγ9Vδ2 T cells, has shown potent anti-tumor activity against several tumour cells *in vitro* and *in vivo* [8-10]. Previous studies reported the use of zoledronate in sensitizing osteosarcoma cells to Vγ9Vδ2 T cell cytotoxicity that may complement current chemotherapies [19,25,26]. Using a murine model of osteolytic breast cancer, we have recently demonstrated that combination treatment of ZOL and Vγ9Vδ2 T cells led to a reduction in breast cancer burden in bone and protected against breast cancer-induced osteolysis [23]. ZOL administration in patients with primary and metastatic bone cancer protects against tumor-associated bone loss and increases bone density, resulting in a reduction in skeletal related events, including fractures and hypercalcemia [27-29]. Given ZOL's preferential uptake and protection in bone, together with its role in triggering proliferation and increasing the cytolytic properties of Vγ9Vδ2 T cells, this study is the first to show the potential for this combination therapy to inhibit osteosarcoma growth in bone and the subsequent cancer-induced bone destruction while also limiting pulmonary metastasis. In line with our previous published data, ZOL alone, inhibited tumor-associated bone loss but had no effect on the growth of the primary tumor. Compared with controls, the infusion of Vγ9Vδ2 T cell alone did not significantly impact tumor burden and tumor-associated bone loss. In contrast, pre-treatment with ZOL, 24 hrs prior to Vγ9Vδ2 T cell infusion, significantly decreased tumor growth within the bone. Moreover, ZOL in combination with Vγ9Vδ2 T cells showed increased protection from osteosarcoma induced bone destruction, such that the tumor-bearing tibia resembled the non-tumor bearing tibia with a significant reduction in tumor growth. Importantly, and consistent with previous findings [23,30], infusion of Vγ9Vδ2 T cells reduced the incidence and tumor burden of lung metastases. It is also interesting to note that in this instance, ZOL treatment alone did not limit lung metastases, suggesting that the combination effect on lung metastases was primarily due to the actions of Vγ9Vδ2 T cells.

A recent study showed that mice pre-treated with ZOL enhanced the cytolytic effects of Vγ9Vδ2 T cells in an orthotopic model of osteoblastic osteosarcoma [25]. In contrast, the present study characterized the effects of ZOL and Vγ9Vδ2 T cells in a model of osteolytic osteosarcoma. While this treatment regimen did not fully eradicate the primary tumor, recent reports suggest that when Vγ9Vδ2 T cells are used in combination with chemotherapy, or cancer-targeting antibodies, the Vγ9Vδ2 T cells display enhanced cytotoxicity [31-33]. These findings suggest that further studies are required to identify the optimal combination therapy that can fully eliminate tumor burden.

Conclusion

In summary, the present study demonstrates that adoptive transfer of *ex vivo*-expanded Vγ9Vδ2 T in combination with ZOL infusion reduces osteosarcoma tumor growth, inhibits tumor-associated bone loss, and limits lung metastases in a murine model of orthotropic osteosarcoma. Therefore, this two-pronged approach may lead to decreased disease severity in patients with osteolytic osteosarcoma.

Disclosure of Interest

The authors declare no conflict of interest.

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