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Original Article

Adoptive transfer of *ex vivo* expanded Vγ9Vδ2 T cells in combination with zoledronic acid inhibits cancer growth and limits osteolysis in a murine model of osteolytic breast cancer

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ABSTRACT

Bone metastases occur in over 75% of patients with advanced breast cancer and are responsible for high levels of morbidity and mortality. In this study, *ex vivo* expanded cytotoxic Vγ9Vδ2 T cells isolated from human peripheral blood were tested for their anti-cancer efficacy in combination with zoledronic acid (ZOL), using a mouse model of osteolytic breast cancer. *In vitro*, expanded Vγ9Vδ2 T cells were cytotoxic against a panel of human breast cancer cell lines, and ZOL pre-treatment further sensitised breast cancer cells to killing by Vγ9Vδ2 T cells. Vγ9Vδ2 T cells adoptively transferred into NOD/SCID mice localised to osteolytic breast cancer lesions in the bone, and multiple infusions of Vγ9Vδ2 T cells reduced tumour growth in the bone. ZOL pre-treatment potentiated the anti-cancer efficacy of Vγ9Vδ2 T cells, with mice showing further reductions in tumour burden. Mice treated with the combination also had reduced tumour burden of secondary pulmonary metastases, and decreased bone degradation. Our data suggests that adoptive transfer of Vγ9Vδ2 T cell in combination with ZOL may prove an effective immunotherapeutic approach for the treatment of breast cancer bone metastases.

Introduction

Breast cancer is one of the most commonly diagnosed cancers in women worldwide. Patients diagnosed with primary breast cancer have higher survival rates compared to those diagnosed with the advanced disease, primarily due to cancer metastases [1]. Bone metastases occur in over 75% of patients with advanced breast cancer, resulting in extensive bone degradation leading to skeletal-related events (SREs) such as hypercalcemia, chronic pain, fracture, spinal cord compression, and impaired mobility, all which greatly affect quality of life [2,3]. Breast cancer bone metastases are predominately osteolytic due to factors secreted by disseminated tumour cells that stimulate osteoclasts [4]. Activated osteoclasts degrade bone and release growth factors from the matrix that further promote tumour growth and bone destruction, perpetuating the 'vicious cycle' of cancer growth and bone destruction [5]. Nitrogen-containing bisphosphonates (nBPs), a class of anti-resorptive drugs, are currently used to inhibit osteoclast-mediated bone degradation in patients with skeletal malignancies, including advanced breast cancer, however, this treatment is only palliative and new therapeutic approaches are required [6,7].

Within the past decade, immunotherapy of cytotoxic gamma delta (γδ) T cells has been gaining momentum as a potential therapeutic approach for targeting cancer. Human γδ T cells comprise a small population (1–10%) of circulating peripheral blood

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lymphocytes [8]. These primarily consist of the Vδ2 chain in combination with Vγ9 (Vγ9Vδ2) which are stimulated and expanded in response of phosphoantigens (PAgs).

Activated Vγ9Vδ2 T cells have the ability to recognise target cells in an MHC-unrestricted manner [9] via detection of PAgs, including isopentenyl pyrophosphate (IPP), an intermediate of the mammalian mevalonate pathway. nBPs, including zoledronic acid (ZOL) inhibit the mevalonate pathway resulting in IPP accumulation which activate and expand Vγ9Vδ2 T cells [10–16].

Due to abnormal upregulation of the mevalonate pathway, tumour cells accumulate PAgs resulting in recognition by Vγ9Vδ2 T cells [17]. Activated Vγ9Vδ2 T cells can then kill cancer cells by releasing Th1 cytokines, including TNF-α (tumour necrosis factor-alpha) and IFN-γ (interferon-gamma) [18–20] and cytolytic granules [10,19–21]. Vγ9Vδ2 T cells also induce target cell death by death receptor/ligand interactions with TRAIL (Apo2L) [21], and FASL (Fas ligand) [11]. As a result, expanded Vγ9Vδ2 T cells exert potent cytotoxicity against a variety of solid and haematological malignancies, *in vitro* and *in vivo* [10–12,15,22,23].

Vγ9Vδ2 T cell immunotherapy has been assessed against a variety of solid and haematological malignancies in early phase clinical trials (reviewed in Ref. [24]). While these trials have deemed Vγ9Vδ2 T cell therapy safe, as a monotherapy the anti-cancer efficacy, especially against advanced tumours has been underwhelming and requires further improvement. In addition to activating Vγ9Vδ2 T cells, ZOL can also sensitise cancer cells to killing by Vγ9Vδ2 T cells both *in vitro* and *in vivo* [13–15,21,25]. Additionally, clinical evidence demonstrates the potential of using Vγ9Vδ2 T cell adoptive transfer in combination with ZOL for the treatment of advanced renal cell carcinoma (RCC), malignant ascites from gastric cancer, and other metastatic tumours [26–28].

As ZOL preferentially localises to the bone, an elegant approach for targeting cancer lesions in the bone has emerged. Discussion in the literature have suggested that nBP administration followed by adoptive transfer of Vγ9Vδ2 T cells would be an ideal two-pronged approach for targeting cancers in the bone [29]. This immunotherapy would allow simultaneous reduction of tumour-associated bone loss in addition to sensitising cancer cells to Vγ9Vδ2 T cell mediated cytotoxicity, inhibiting the vicious cycle of bone destruction and cancer growth. To date, adoptive transfer of Vγ9Vδ2 T cells alone or in combination with ZOL to specifically target cancers in the bone has not been fully investigated. In this study, we used a murine model of osteolytic breast cancer, where breast cancer cells were implanted directly into the tibia in NOD/SCID mice. We showed for the first time, that Vγ9Vδ2 T cells localised to osteolytic breast cancer lesions growing in the bone and that multiple infusions of Vγ9Vδ2 T cells slowed tumour growth. We also showed that ZOL potentiated the anti-cancer efficacy of Vγ9Vδ2 T cells, decreased tumour burden in the bone, inhibited tumour-associated osteolysis, and decreased lung metastases tumour burden.

Materials and methods

Cells and reagents

ZR75 and T47D human breast cancer cell lines were obtained from American Type Culture Collection. The MDA-MB231 human breast cancer derivative cell line MDA-MB231-TXSA was kindly provided by Dr. Toshiyuki Yoneda (University of Texas Health Science Centre, San Antonio, Texas). MDA-MB231-TXSA expressed GFP and luciferase produced by retroviral expression of the SFG-NES-TGL vector, as previously described [30]. All cell lines were cultured in 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 generously provided by Novartis Pharma AG.

Ex vivo expansion of Vγ9Vδ2 T cells

Informed consent was obtained prior to collection of peripheral blood from healthy adult donors. PBMC were isolated immediately via density gradient centrifugation using Lymphoprep™ (Axis Shield, Norway) following manufacturer's instructions. PBMCs were resuspended to 1×10^6/mL in CTS™ OpTmizer™ T Cell Expansion SFM (Life Technologies, Australia) supplemented with OpTmizer™ T cell Expansion Supplement (1:38 dilution) (Life Technologies, Australia), 10% heat-inactivated FBS (HI-FBS), 100 IU/mL penicillin, 100 μg/mL streptomycin, 2 mmol L-glutamine (Life Technologies, Australia), 25 mM HEPES, 0.1% β-mercaptoethanol (Sigma–Aldrich, USA), 100 IU/mL recombinant human interleukin 2 (rhIL-2) (BD Pharmingen, USA) and activated with 5 μM ZOL, and seeded into 6-well plates. Cell culture density was maintained at $1–2 \times 10^6$ cells/mL and replenished with fresh medium containing 100 IU/mL rhIL-2 only (without ZOL) every 2–3 days. Following 7–8 days of culture cells were collected and enriched as described below.

Enrichment of Vγ9Vδ2 T cells

Ex vivo expanded Vγ9Vδ2 T cells were enriched prior to *in vitro* and *in vivo* experiments using negative selection MACS with the TCR γ/δ+ T cell Isolation Kit (human) (Miltenyi Biotec, Germany). Cell viability and total cells numbers after enrichment were assessed using trypan blue exclusion. Percentage of Vγ9Vδ2 T cells were determined by flow cytometry using PeCy5 conjugated anti-CD3 (clone UCHT1) (eBioscience, San Diego, CA, USA) and FITC conjugated anti-Vγ9 TCR from BD Biosciences (San Jose, CA, USA). Analysis was performed on the BD FACSCanto II Flow Cytometer (San Jose, CA, USA). Percentages of Vγ9Vδ2 T cells were identified by gating on the lymphocyte population using forward scatter/side scatter then on $V\gamma9^+$ $CD3^+$ double positive cells. After enrichment, Vγ9Vδ2 T cell viability was >95%, and the percentage of Vγ9Vδ2 T cells was consistently >97%.

Cell cytotoxicity assay

Cytotoxicity of Vγ9Vδ2 T cells against breast cancer cell lines was assessed using a standard lactate dehydrogenase (LDH) release assay (CytoTox 96® Non-Radioactive Cytotoxicity Assay; Promega, USA). Briefly, 1×10^4 target cells were seeded in triplicate in a 96-well microtiter plate and allowed to adhere overnight. Target cells were then treated with or without 25 μM ZOL for 24 h, and then co-cultured with Vγ9Vδ2 T cells at 1:1, 5:1 and 10:1 effector:target (E:T) ratio, with Vγ9Vδ2 T cells as the effector, and cancer cells as the target. After incubation for 9 h at 37 °C, 50 μL of supernatant was assayed for LDH activity following the manufactures protocol. The appropriate controls were prepared and cytotoxicity was calculated as:

$$\%\,Cytotoxicity = \frac{experimental\ release - effector\ spontaneous\ release - target\ spontaneous\ release}{targetmaximumrelease - targetspontaneousrelease} \times 100$$

Cell viability assay

MDA-MB231-TXSA cells expressed luciferase, which was the basis for a luciferase activity viability assay using Dual Luciferase® Reporter Assay kit (Promega, Madison, WI, USA). Briefly, 1×10^4 luciferase-tagged target cells were seeded in triplicate in a 96-well microtiter plate and allowed to adhere overnight. Cells were then treated with or without 25 μM ZOL for 24 h, and then co-cultured with Vγ9Vδ2 T cells at 1:1, 5:1 and 10:1 E:T ratios. After 24 h incubation, media was removed and cells were washed in PBS, then lysates were prepared and analysed following the manufacturers protocol. Viability was calculated as:

$$\%\,Viability = \frac{experimental\ value}{untreated\ control\ value} \times 100$$

Measurement of DEVD-caspase activity

DEVD-caspase activity was assayed by cleavage of zDEVD-AFC (z-asp-glu-val-asp-7-amino-4-trifluoro-methyl-coumarin), a fluorogenic substrate based on the peptide sequence at the caspase-3 cleavage site of poly (ADP-ribose) polymerases