Title: **Zoledronic acid renders human M1 and M2 macrophages susceptible to Vδ2+ γδ T cell cytotoxicity in a perforin dependent manner**

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Abstract

Vδ2+ T cells are a subpopulation of γδ T cells in humans that are cytotoxic towards cells which accumulate isopentenyl pyrophosphate. The nitrogen-containing bisphosphonate, zoledronic acid (ZA), can induce tumour cell lines to accumulate isopentenyl pyrophosphate, thus rendering them more susceptible to Vδ2+ T cell cytotoxicity. However, little is known about whether ZA renders other, non-malignant cell types susceptible. In this study we focussed on macrophages (Mφs), as these cells have been shown to take up ZA. We differentiated peripheral blood monocytes from healthy donors into Mφs, and then treated them with IFN-γ or IL-4 to generate M1 and M2 Mφs, respectively. We characterised these Mφs based on their phenotype and cytokine production, and then tested whether ZA rendered them susceptible to Vδ2+ T cell cytotoxicity. Consistent with the literature, IFN-γ-treated Mφs expressed higher levels of the M1 markers CD64 and IL-12p70; whereas, IL-4-treated Mφs expressed higher levels of the M2 markers CD206 and chemokine (C-C motif) ligand 18. When treated with ZA, both M1 and M2 Mφs became susceptible to Vδ2+ T cell cytotoxicity. Vδ2+ T cells expressed perforin and degranulated in response to ZA-treated Mφs as shown by mobilisation of CD107a and CD107b to the cell surface. Furthermore, cytotoxicity towards ZA-treated Mφs was sensitive—at least in part—to the perforin inhibitor concanamycin A. These findings suggest that ZA can render M1 and M2 Mφs susceptible to Vδ2+ T cell cytotoxicity in a perforin dependent manner, which has important implications regarding the use of ZA in cancer immunotherapy.
Key words

γδ T cell; Vδ2+ T cell; macrophage; zoledronic acid; cytotoxicity.
Précis

This study identifies a novel effect of zoledronic acid on Mφs and provides a potential mechanism of action that may help the future development of this drug in cancer immunotherapy.
**Abbreviations**

| Abbreviation | Description |
|--------------|-------------|
| CCL          | chemokine (C-C motif) ligand |
| CFSE         | carboxyfluorescein succinimidyl ester |
| CMA          | concanamycin A |
| FSC          | forward scatter |
| G            | gate |
| IPP          | isopentenyl pyrophosphate |
| LSD          | least significant difference |
| Mϕ           | macrophage |
| MFI          | mean (arithmetic) fluorescence intensity |
| NBP          | nitrogen-containing bisphosphonate |
| SD           | standard deviation |
| SSC          | side scatter |
| TAM          | tumour-associated macrophage |
| ZA           | zoledronic acid |
Introduction

Human peripheral blood contains a subpopulation of γδ T cells that express TCRs composed of Vγ9 and Vδ2 subunits. These cells—referred to here as Vδ2+ T cells—typically represent 0.5–5 percent of peripheral blood T cells, and exert potent cytotoxicity against their target cells.

Vδ2+ T cells detect intermediates of isoprenoid biosynthesis, namely isopentenyl pyrophosphate (IPP) and (E)-4-hydroxy-3-methyl-but-2-enyl pyrophosphate. IPP is generated by the endogenous mevalonate pathway as well as the exogenous 1-deoxy-D-xylulose-5-phosphate pathway; whereas, (E)-4-hydroxy-3-methyl-but-2-enyl pyrophosphate is generated by the 1-deoxy-D-xylulose-5-phosphate pathway only [1]. The mevalonate pathway is often dysregulated in malignant and infected cells, resulting in accumulation of IPP and increased susceptibility to Vδ2+ T cell cytotoxicity [2, 3]. Moreover, certain cells accumulate IPP when exposed to the nitrogen-containing bisphosphonate (NBP), zoledronic acid (ZA) [4], a synthetic drug that inhibits an enzyme of the mevalonate pathway called farnesyl pyrophosphate synthase [5]. Although the precise mechanism of IPP and (E)-4-hydroxy-3-methyl-but-2-enyl pyrophosphate recognition by Vδ2+ T cells has yet to be determined, evidence suggests that it is TCR-dependent and involves butyrophilin 3A1 [6].

ZA is typically used to treat complications associated with excessive bone resorption in diseases such as osteoporosis, Paget’s disease and metastatic bone disease [7]. In terms of its mode of action, ZA binds to bone and disrupts the activity of bone remodelling cells called osteoclasts [8]. ZA also has potential as an immunotherapy for cancer, the proof of concept for which has already been demonstrated in clinical trials [9-11]. Although in cancer its mode of action is poorly understood, experiments in vitro have shown that tumour cell lines from a broad range of haematological and solid malignancies become more susceptible to Vδ2+ T cell cytotoxicity when exposed to ZA, suggesting a role for Vδ2+ T cells [12-14]. However, the capacity for ZA to induce susceptibility in other, non-malignant cell types is poorly characterised, and could provide insight that helps to better understand the effects of this drug and improve its clinical application. In this study we have focussed on macrophages (denoted here as Mϕs) because these cells have been shown recently to take up NBPs in vivo [15] and are implicated in the progression of cancer [16].
Mϕs are tissue-resident phagocytic cells that play a critical role in tissue repair as well as immunity against pathogenic infection and malignant transformation [17]. Mϕs display functional plasticity that is intricately linked to their surrounding microenvironment [18]. Researchers have categorised the different functional states of Mϕs according to their capacity to either promote inflammation or suppress it. At one end of the spectrum are pro-inflammatory Mϕs, also referred to as M1 or classically activated Mϕs, and at the other end are anti-inflammatory Mϕs, also known as M2 or alternatively activated Mϕs [19]. IFN-γ and IL-4 have been identified as key drivers of these opposing M1 and M2 phenotypes, respectively [19].

As part of our ongoing studies into how ZA stimulates anti-tumour responses in Vδ2+ T cells, we identified a previously unexplored effect involving Vδ2+ T cell targeting of myeloid cells. Recently, we showed that ZA can render peripheral blood monocytes susceptible to Vδ2+ T cell cytotoxicity in vitro [20]. In a subsequent study by Junankar et al, tumour-associated Mϕs (TAMs) in breast cancer were identified as important targets for NBPs in vivo [15]. Therefore, we further explored the concept of Vδ2+ T cell targeting of myeloid cells, and found that ZA can render M1 and M2 Mϕs susceptible to Vδ2+ T cell cytotoxicity. Furthermore, we found that Vδ2+ T cell cytotoxicity towards ZA-treated Mϕs was dependent—at least in part—on perforin. This novel insight into the interplay between Vδ2+ T cells and Mϕs has important implications regarding the use of ZA in cancer immunotherapy.
Materials and Methods

**PBMC isolation**

Anonymised leukocyte cones from healthy donors were obtained from the National Health Service blood transfusion unit at St. George’s Hospital, London. PBMCs were isolated by density adjusted centrifugation using Histopaque-1077 (Sigma-Aldrich). RBCs were lysed with ammonium chloride solution and platelets removed by slow-speed centrifugation. PBMCs were resuspended at 2×10^7 cells/ml of freezing medium (45% RPMI-1640, 45% FBS and 10% DMSO; all from Sigma-Aldrich) and frozen at −80°C in Mr Frosty freezing containers (Thermo Scientific) prior to transferring them to liquid nitrogen.

**Cell culture**

All cell culture was carried out in a humidified incubator at 37°C with 5% CO₂. To generate MΦs, monocytes were isolated from PBMCs using CD14 microbeads according to the manufacturer’s instructions (Miltenyi Biotec). Monocytes were resuspended in serum-free medium (RPMI-1640 containing 2mM L-glutamine, 100units/ml penicillin and 100μg/ml streptomycin; all from Sigma-Aldrich) at a density of 3.8×10^6 cells/ml, and 200μls, 2mls or 5mls of cell suspension added per well of 96-well, 12-well or 6-well tissue culture plates, respectively (Thermo Scientific). Monocytes were cultured for 2 hours, after which time the majority of cells were adherent to the tissue culture plate. This process is known to activate monocytes and initiate the macrophage colony-stimulating factor production required for MΦ differentiation [21]. The adherent monocytes were then cultured for 10 days in complete medium (RPMI-1640 containing 10% FBS, 2mM L-glutamine, 100units/ml penicillin and 100μg/ml streptomycin), after which time the monocytes had differentiated into MΦs, as indicated by the morphological changes and plastic adherence observed using light microscopy. M1 and M2 MΦs were generated by adding 25ng/ml of recombinant human IFN-γ or IL-4 (R and D Systems), respectively, on day 7. MΦs that had not been treated with IFN-γ or IL-4 (designated M0s) were used as controls throughout. 10μM ZA (Sigma-Aldrich) was added to the MΦs on day 9. To generate pure populations of Vδ2+ T cells, PBMCs were resuspended at 2×10^6 cells/ml of complete medium containing 1μM ZA and 5ng/ml recombinant human IL-2 (R and D Systems), and 250μls of cell suspension added per well of 96-well round-bottomed tissue culture plates (Thermo Scientific). The cells were cultured for 9 days and fed every 2–3 days with...
fresh medium containing 5ng/ml IL-2. Dead cells and non-γδ T cells were depleted sequentially using
dead cell removal kits and TCRγδ negative isolation kits according to the manufacturer’s instructions
(Miltenyi Biotec). Purity of Vδ2+CD3+ cells was assessed by flow cytometry using PE-conjugated mouse
anti-human Vδ2 (clone 123R3; Miltenyi Biotec) and PerCP-conjugated mouse anti-human CD3 (clone
SK7; Biolegend) or FITC-conjugated mouse anti-human CD3 (clone HIT3a; Becton Dickinson). For one
donor, a high percentage of Vδ1+CD3+ cells was detected post isolation, and so Vδ1+ cells were
depleted using allophycocyanin-conjugated recombinant human anti-Vδ1 (clone REA173; Miltenyi
Biotec) and anti-allophycocyanin microbeads according the manufacturer’s instructions (Miltenyi Biotec;
 supplementary Fig. 1). We speculate that this donor’s PBMCs had a particularly high percentage of
Vδ1+ T cells prior to ZA and IL-2 stimulation and/or their Vδ1+ T cells underwent bystander expansion
in response to ZA and IL-2.

Flow cytometry

Day 10 Mφs in 6-well tissue culture plates were washed twice in PBS (Sigma-Aldrich) and
cultured for 15 minutes in PBS containing 0.25% trypsin (Life Technologies) and 2mM EDTA (Sigma-
Aldrich). Cells were detached by repeated pipetting and then washed in complete medium to deactivate
the trypsin. Mφs were resuspended in flow cytometry buffer (PBS with 1% BSA and 0.09% sodium
azide; all from Sigma-Aldrich) containing either FITC-conjugated mouse anti-human CD64 (clone 10.1;
Becton Dickinson) or PE-conjugated mouse anti-human CD206 (clone 19.2; Becton Dickinson).
Matched isotype controls were used to determine the amount of background expression. After 10
minutes at room temperature, cells were washed in flow cytometry buffer and fixed in CellFIX (Becton
Dickinson). Perforin expression in Vδ2+ T cells was assessed in PBMCs cultured with ZA and IL-2 for
0, 1 and 9 days as described in the cell culture section. Cells were resuspended in flow cytometry buffer
containing PE-conjugated mouse anti-human Vδ2 (clone 123R3; Miltenyi Biotec) and PerCP-
conjugated mouse anti-human CD3 (clone SK7; Biolegend). After 10 minutes at room temperature,
cells were washed in flow cytometry buffer and simultaneously fixed and permeabilised using
Cytofix/Cytoperm (Becton Dickinson) according to manufacturer’s instructions. Cells were washed and
resuspended in Perm/Wash buffer (Becton Dickinson), and then labelled with FITC-conjugated mouse
anti-human perforin (clone δG9; Becton Dickinson) or matched isotype controls. After 10 minutes at
room temperature, cells were washed in Perm/Wash buffer and resuspended in flow cytometry buffer.
Samples were acquired on an LSR II flow cytometer (Becton Dickinson) and analysed using FlowJo software. All comparatively analysed samples were acquired on the same day except for the time course of perforin expression where day 0, 1 and 9 samples were acquired on different days. The mean fluorescence intensity (MFI) values stated throughout are arithmetic means.

**ELISAs**

Day 10 Mφs in 12-well tissue culture plates were washed twice in PBS and cultured overnight in complete medium (1ml/well) with or without 100ng/ml LPS (*E.coli* 0127:B8; Sigma-Aldrich). The concentration of IL-12p70 and chemokine (C-C motif) ligand (CCL) 18 within cell-free culture supernatants was determined using DuoSet ELISA kits according to the manufacturer’s instructions (R and D Systems). Optical densities at 450nm were determined using a microplate reader (Dynex), and concentrations were extrapolated from standard curve data using a four parameter logistic model generated by GraphPad Prism 6 (GraphPad Software). Standard curves were 31.25–2000pg/ml for IL-12p70, and 7.8125–500pg/ml for CCL18.

**Carboxyfluorescein succinimidyl ester/Zombie-NIR cytotoxicity assay**

Detaching the Mφs from the tissue culture plates prior to performing the cytotoxicity assays resulted in poor viability; therefore, cytotoxicity was assessed by adding Vδ2⁺ T cells directly to adherent Mφs. Day 10 Mφs in 12-well tissue culture plates were washed twice in PBS and then cultured for 20 minutes in PBS containing 1μM carboxyfluorescein succinimidyl ester (CFSE; Life Technologies). Mφs were washed three times in complete medium and then cultured overnight with or without 1.52×10⁶ autologous Vδ2⁺ T cells per well in 2mls complete medium to obtain an E:T ratio of 2:1 based on the initial seeding density of monocytes. For some experiments Vδ2⁺ T cells were pre-treated for 2 hours with or without 100ng/ml concanamycin A (CMA; Abcam) or DMSO, then washed three times in complete medium prior to being cultured with Mφs. Non-adherent cells were collected and adherent cells detached from the tissue culture plates as described in the flow cytometry section. All cells were washed in PBS and then labelled with Zombie-NIR live/dead discrimination dye according to the manufacturer's instructions (Biolegend). Zombie-NIR binds to amine groups on proteins but does not penetrate an intact plasma membrane. Live cells have relatively low expression because only cell
surface proteins are available for binding; whereas, dead cells exhibit higher levels of expression because their compromised plasma membrane permits binding to both extracellular and intracellular proteins. After 15 minutes at room temperature, cells were washed in complete medium and fixed in CellFix. Samples were acquired on an LSR II flow cytometer and analysed using FlowJo software. All comparatively analysed samples were acquired on the same day.

**CD107 mobilisation assay**

Day 10 Mφs in 96-well tissue culture plates were washed three times in PBS and then cultured for 5 hours with 1.52×10^5 autologous Vδ2^+ T cells per well in 200μls complete medium to obtain an E:T ratio of 2:1 based on the initial seeding density of monocytes. Allophycocyanin-conjugated mouse anti-human CD107a (clone H4A3; Biolegend) and FITC-conjugated mouse anti-human CD107b (clone H4B4; Biolegend) or matched isotype controls were added directly to the wells at the start of the co-culture along with 1μg/ml of monensin to neutralise intracellular acidity. Cells were then collected and labelled with PE-conjugated mouse anti-human Vδ2 (clone 123R3; Miltenyi Biotec) and PerCP-conjugated mouse anti-human CD3 (clone SK7; Biolegend) as described in the flow cytometry section. Samples were acquired on an LSR II flow cytometer and analysed using FlowJo software. All comparatively analysed samples were acquired on the same day.

**Statistical analyses**

Data in Fig. 1b, 1c, 3b, 3d and 4c was analysed by repeated measures one-way or two-way ANOVA, and comparisons between means carried out using either Tukey’s or Sidak’s multiple comparison tests (GraphPad Prism 6). *, **, *** and **** were used to indicate p values of <0.05, <0.01, <0.001 and <0.0001, respectively. Gaussian distributions were assumed. Data in Fig. 2b was a three-way (3 × 2 × 2) factorial design repeated six times using cells from six different donors. The three factors were Mφ type (M0, M1 and M2), ±ZA and ±Vδ2 cells. Data in Fig. 4b was a three-way (3 × 2 × 4) factorial design repeated five times using cells from five different donors. The three factors were Mφ type (M0, M1 and M2), ±ZA and ±Vδ2 cells (−Vδ2, +Vδ2, +Vδ2[DMSO] and +Vδ2[CMA]). Data in Fig. 2b and 4b was analysed by three-way ANOVA, and comparisons between means carried out using Fisher’s Least Significant Difference (LSD; Genstat 18). Assumptions underlying the analysis were checked using the
diagnostic plots produced by the software. LSDs at the 5, 1 and 0.1% level are depicted by black
intervals, and differences in the means that were greater than this interval were deemed significant to
an equivalent p value of <0.05, <0.01 and <0.001, respectively.
Results

ZA did not alter M1 or M2 markers on Mφs

We differentiated monocytes from the peripheral blood of healthy donors into Mφs, and treated them with IFN-γ or IL-4 to generate M1 and M2 Mφs, respectively. We then characterised these Mφs based on their expression of markers for M1 Mφs (CD64 and IL-12p70) and M2 Mφs (CD206 and CCL18) [19, 22]. M1 Mφs had upregulated expression of CD64; whereas, M2 Mφs had downregulated CD64 and upregulated CD206 (Fig. 1a and b). Although, statistically, we observed significantly higher levels of CD206 on M1 Mφs compared with M0 Mφs in terms of percentage expression, this was not consistent for all donors and not statistically significant in terms of relative MFI (Fig. 1b). Mφs were then cultured overnight with or without LPS to measure production of IL-12p70 and CCL18, respectively. M1 Mφs produced more IL-12p70 than M0 and M2 Mφs; whereas, M2 Mφs produced more CCL18 than M0 and M1 Mφs (Fig. 1c). We also tested whether ZA—added for the last 18 hours of culture—had any effect on these markers, and found little or no difference between untreated and ZA-treated Mφs (Fig. 1). Taken together, this data validates our protocol for generating M1 and M2 Mφs, and shows that ZA does not alter the M1 and M2 profile of the Mφs in this system.

ZA rendered M1 and M2 Mφs susceptible to Vδ2+ T cell cytotoxicity

To obtain sufficient cell numbers for cytotoxicity assays, we stimulated Vδ2+ T cell expansion prior to isolation. Vδ2+ T cell expansion was observed in PBMCs treated with ZA and IL-2 for 9 days, as shown by increased frequencies of Vδ2-CD3+ cells (supplementary Fig. 2). Vδ2+ T cells were purified by sequentially depleting dead cells and non-γδ T cells (mean±standard deviation (SD) for the percentage of Vδ2-CD3+ cells from four donors = 97.2±1.8; supplementary Fig. 2). The percentage of Vδ2-CD3+ cells at day 0 and day 9 pre depletion of dead cells and non-γδ T cells was not assessed routinely; however, purities at day 9 post depletion were assessed for all isolations performed in this study (mean±SD for 14 isolations = 97.7±1.8). We conducted preliminary experiments to determine the optimal E:T ratio and ZA concentration for Vδ2+ T cell-mediated cytotoxicity against ZA-treated Mφs (supplementary Fig. 3). These experiments showed Vδ2+ T cell cytotoxicity and degranulation against Mφs treated with 10μM but not 1μM ZA (supplementary Fig. 3). Furthermore, they showed marked killing at the lowest E:T ratio of 2:1 (supplementary Fig. 3). Using the 10μM concentration of ZA and 2:1
E:T ratio, we found that ZA had little or no effect on Mφ viability in the absence of Vδ2+ T cells, and Vδ2+ T cells did not induce cell death in Mφs that had not been treated with ZA (Fig. 2a and b). However, there was a marked increase in the amount of cell death in Mφs that were pre-treated with ZA and then cultured with Vδ2+ T cells (Fig. 2a and b). Although, statistically, Vδ2+ T cell-mediated killing of ZA-treated M1 Mφs was significantly higher than that of M0 Mφs, the difference was relatively small and no statistically significant difference was found between M1 and M2 Mφs (Fig. 2b). These results suggest that Vδ2+ T cells are cytotoxic towards ZA-treated Mφs irrespective of their M0, M1 and M2 phenotype.

Vδ2+ T cells expressed perforin and degranulated when cultured with ZA-treated Mφs

Perforin has been shown previously to play a role in γδ T cell cytotoxicity towards tumour cell lines [23, 24]; therefore, we tested whether perforin contributes to Vδ2+ T cell cytotoxicity towards ZA-treated Mφs. We measured perforin expression by Vδ2+ T cells before, during and after expansion with ZA and IL-2. We found that, although resting Vδ2+ T cells expressed little or no perforin, it was markedly upregulated after one day of culture with ZA and IL-2 (Fig. 3a and b). After 9 days of culture with ZA and IL-2, perforin was downregulated but still expressed by Vδ2+ T cells (Fig. 3a and b). We also measured perforin expression in expanded and isolated Vδ2+ T cells from six donors, and found consistent expression in terms of percentage expression (means±SD = 1.2±0.5 vs. 23.9±7.6 for isotype and test, respectively) and MFI (means±SD = 227.8±15.2 vs. 490.7±104.6 for isotype and test, respectively). To determine if Vδ2+ T cells release perforin when cultured with ZA-treated Mφs, we measured the mobilisation of lysosomal-associated membrane protein 1 and 2 (i.e. CD107a and CD107b) to the surface of Vδ2+ T cells. CD107a—and to a lesser extent CD107b—was expressed on Vδ2+ T cells that were isolated from PBMCs after 9 days of culture with ZA and IL-2 (Fig. 3c). This may represent residual CD107 expression from the monocyte-dependent degranulation that is induced when PBMCs are exposed to ZA [20]. Vδ2+ T cells upregulated expression of CD107a and CD107b on their cell surface when cultured with ZA-treated Mφs compared with untreated Mφs (Fig. 3c and d). This data suggests that Vδ2+ T cells express perforin and degranulate in response to ZA-treated Mφs, thus implicating a role for perforin in Vδ2+ T cell cytotoxicity towards ZA-treated Mφs.
**Vδ2+ T cell cytotoxicity towards ZA-treated MФs was sensitive to concanamycin A**

To explore further the potential role of perforin in Vδ2+ T cell cytotoxicity towards ZA-treated MФs, we repeated the cytotoxicity assays shown in Fig. 2, but this time pre-treated Vδ2+ T cells with the H+-ATPase inhibitor CMA. CMA blocks acidification of cytolytic granules, which inhibits perforin-mediated but not Fas ligand-mediated cytotoxicity [25]. We found that pre-treating Vδ2+ T cells with CMA reduced their cytotoxicity towards MФs compared with DMSO controls (Fig. 4a and b). We calculated the percentage inhibition for Vδ2+ T cell cytotoxicity towards M0, M1 and M2 MФs, and found that Vδ2+ T cell cytotoxicity towards M0 MФs was more sensitive to CMA than towards M1 MФs (Fig. 4c). To determine whether CMA had an effect on Vδ2+ T cell viability, we applied a gate to CFSE− cells and calculated the percentage of Zombie-NIRlow cells (supplementary Fig. 4a). There was a discernible reduction in Vδ2+ T cell viability in the presence of ZA-treated MФs compared with untreated MФs; however, there was little or no difference in Vδ2+ T cell viability between the CMA and DMSO treatment groups (supplementary Fig. 4b). These findings suggest that Vδ2+ T cell cytotoxicity towards ZA-treated MФs is sensitive—at least in part—to CMA, thus implicating a role for perforin.
**Discussion**

Vδ2+ T cells in the peripheral blood of humans are regarded as sentinels against infection [26] and malignant transformation [27]. They express the inflammatory homing receptors chemokine (C-C motif) receptor 5 and chemokine (C-X-C motif) receptor 3 [28], and thus infiltrate sites of infection [29] as well as the inflammatory microenvironment of diseased tissues such as tumours [30, 31]. Mφs are abundant in these tissues, and are likely to interact closely with infiltrating Vδ2+ T cells. We explored the potential interaction between Vδ2+ T cells and Mφs *in vitro*, and found that ZA can render M1 and M2 Mφs susceptible to Vδ2+ T cell cytotoxicity in a perforin dependent manner.

ZA has a high affinity for hydroxyapatite [32], and thus binds rapidly to bone following i.v. infusion [33]. Therefore, the Mφs most likely to be exposed to ZA are those associated with bone and/or the surrounding tissues; for example, the TAMs in bone-related cancers such as osteosarcoma, myeloma and secondary bone metastases associated with cancers of the prostate, lung and breast. Following i.v. infusion, NBPs may also reach tissues other than bone. Intravital imaging in a murine model of breast cancer showed that a fluorescently labelled NBP—given by i.v. injection—leaked from the vasculature of mammary tumours and bound rapidly to granular microcalcifications, which were subsequently engulfed by TAMs [15]. The NBP was not retained in cells other than Mφs, nor was it retained in B16 tumours, which lack microcalcifications [15]. This study suggests that calcified tissues other than bone can also accumulate NBPs [15]. The lack of cytotoxicity and degranulation at 1μM ZA that was observed in our preliminary optimisation experiments suggests that the Mφs most likely to be targeted by Vδ2+ T cells following ZA treatment are those associated with calcified tissues where the drug is likely to accumulate, which has important implications regarding the *in vivo* effects of this drug. It is worth noting that uptake of ZA by Mφs *in vivo* may be markedly different using other methods of delivery such as liposome or nanoparticle encapsulation [34, 35] and localised injection. At the cellular level, experiments conducted *in vitro* suggest that ZA is taken up by myeloid cells such as monocytes, Mφs and osteoclasts via the process of fluid phase endocytosis [36, 37].

ZA inhibits farnesyl pyrophosphate synthase of the mevalonate pathway, which has been shown *in vitro* to induce apoptosis directly in the murine Mφ-like cell line J774.2 [38]. A potential mechanism for this
effect is accumulation of the pro-apoptotic analogue of ATP, Apppl, which has been reported to accumulate in ZA-treated cells such as osteoclasts and MCF-7 cells [4]. Interestingly, ZA did not affect the viability of Mϕs in our experiments; however, we used relatively short exposure times and did not look at markers of early stage apoptosis such as surface expression of phosphatidyl serine. Inhibition of farnesyl pyrophosphate synthase may also modulate the differentiation and function of Mϕs. For example, when monocyte-derived M2 Mϕs were differentiated in the presence of ZA, they had reduced expression of CD206 and IL-10, and an impaired capacity to promote angiogenesis and tumour cell invasion [39]. ZA also inhibited tumour growth in a murine model of cervical cancer, which correlated with reduced angiogenesis and decreased production of matrix metalloproteinase 9 by Mϕs proximal to and associated with tumours [40]. Furthermore, ZA reduced the onset and growth of tumours in a murine model of breast cancer, which correlated with reduced vascularisation of the tumour, reduced numbers of TAMs, and repolarisation of TAMs from an M2 to M1 phenotype [41]. Taken together, these studies suggest that ZA can modulate the differentiation of Mϕs towards an M1 phenotype. To the best of our knowledge, Vδ2+ T cell targeting of ZA-treated M1 and M2 Mϕs—as suggested by our data—is previously unreported and broadens our understanding of the effects of ZA on Mϕs. Importantly, mice do not develop the Vδ2+ T cell subset that responds to ZA-induced accumulation of IPP because they lack the gene for butyrophilin 3A1 [42], thus highlighting the importance of using human cells for this study.

Our data suggests that ZA has the potential to kill M1 and M2 Mϕs indirectly within tissues that are exposed to the drug and infiltrated by Vδ2+ T cells. Tumours contain an abundant population of Mϕs, which typically express M2 markers and correlate with a poor prognosis [43]. In breast cancer, CCL18 production by TAMs promotes angiogenesis and thus supports tumour growth and dissemination [44]. Furthermore, M2 Mϕs in the bone marrow of multiple myeloma patients have been shown to protect malignant cells from chemotherapy-induced apoptosis [45, 46]. In contrast, osteosarcomas can contain relatively high percentages of M1 Mϕs, which are associated with reduced metastases and improved survival [47]. The potential for ZA to render Mϕs susceptible to Vδ2+ T cells may be beneficial or detrimental depending on which type of Mϕs are present in the tumour. For example, it may be beneficial in patients with breast cancer or myeloma and could explain the promising responses to ZA reported for clinical trials in these cancer types [48, 49]; whereas, it may be counterproductive in osteosarcoma.
It is important to note that our study has focused on the killing capacity of activated Vδ2+ T cells. Although it would be interesting to compare the cytotoxicity of resting and activated Vδ2+ T cells, the relatively low frequency of Vδ2+ T cells in peripheral blood meant that we were unable to isolate the number of resting Vδ2+ T cells required to perform the cytotoxicity assays used in this study. Whether or not i.v. infusion of ZA—combined with i.v. or s.c. IL-2—can activate peripheral blood Vδ2+ T cells in vivo is a point of contention. Current hypotheses state that peripheral blood monocytes take up ZA following i.v. infusion and subsequently activate Vδ2+ T cells [37]; indeed, proliferation and/or differentiation of peripheral blood Vδ2+ T cells has been reported in some patients receiving ZA and IL-2 [9-11]. However, Vδ2+ T cell responses were not observed in all patients [50], and it is unclear whether this is due to lack of activation or detection. Importantly, Vδ2+ T cells that are pre-activated may be more cytotoxic than resting, and thus ZA-induced targeting of MΦs by Vδ2+ T cells in vivo may be suboptimal in patients for which ZA and IL-2 treatment fails to activate their circulating Vδ2+ T cells, thus highlighting the importance of effective Vδ2+ T cell priming in the periphery.

In our study, Vδ2+ T cell cytotoxicity towards M0, M1 and M2 MΦs was sensitive—at least in part—to the perforin inhibitor CMA, thus implicating a role for perforin [25]. Interestingly, CMA did not inhibit cytotoxicity completely, and the degree of inhibition varied between the different types of MΦ; specifically, Vδ2+ T cell cytotoxicity towards M0 MΦs was more sensitive to CMA than towards M1 MΦs. If, in our assays, CMA blocked perforin completely, our data would suggest that other mechanisms of cell-mediated cytotoxicity are involved, and that the contribution of perforin versus other mechanisms of cytotoxicity varies between the different types of MΦ. Indeed, Vδ2+ T cells have been shown to kill target cells through the expression of Fas ligand and TRAIL [51]. However, if perforin blockade was incomplete, the variation in sensitivity to CMA that was observed between the different types of MΦ could also be attributed to differences in their susceptibility to perforin-mediated killing under conditions of suboptimal perforin activity. Nonetheless, our data suggests that perforin plays a role, which provides a useful mechanistic marker for exploring this concept in vivo.
In conclusion, this study sheds light on a potential interaction between Vδ2+ T cells and Mφs following ZA treatment, and suggests a mechanism of action for this drug that may help its future development in cancer immunotherapy.
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Conflict of interest

The authors declare that they have no conflict of interest.
Figure legends

**Figure 1:** Characterisation of M1 and M2 Mϕs treated with or without ZA. (a) and (b) Flow cytometry was used to measure the expression of CD64 and CD206 on M0, M1 and M2 Mϕs treated with (orange) or without (blue) ZA for the last 18 hours of culture. (a) Representative flow cytometry plots from one of six donors (~ZA). Dead cells and debris were excluded based on forward scatter (FSC) and side scatter (SSC) using gate (G) 1. Unfilled overlays = test, filled overlays = isotype. Numbers on the plots are percentage of cells within the marker. (b) Individual data points and means for six donors. Test MFI for the total G1 population were divided by isotype controls to obtain relative MFI. (c) M0, M1 and M2 Mϕs treated with (orange) or without (blue) ZA for the last 18 hours of culture were cultured overnight in fresh medium with or without 100ng/ml LPS. The concentration of IL-12p70 and CCL18 in culture supernatants was measured using ELISAs. Data for IL-12p70 is in the presence of LPS; whereas, data for CCL18 is in the absence of LPS. Individual data points and means for six donors are shown. For (b) and (c), data was analysed by repeated measures two-way ANOVA, and comparisons between means carried out using Tukey's multiple comparison tests. *** and **** indicate p values of <0.001 and <0.0001, respectively. Statistical differences for comparisons within the +ZA (orange) data sets are not shown.

**Figure 2:** Vδ2+ T cell cytotoxicity towards ZA-treated M1 and M2 Mϕs. M0, M1 and M2 Mϕs treated with or without ZA for the last 18 hours of culture were labelled with CFSE and cultured overnight with or without autologous Vδ2+ T cells. Flow cytometry was then used to measure Zombie-NIR expression. (a) Representative flow cytometry contour plots from one of six donors showing the gating strategy used to determine Zombie-NIR expression in M0 Mϕs. Mϕs were gated based on FSC and SSC using G1. CFSE+ cells within G1 were gated using G2. The percentage of Zombie-NIR\textsuperscript{high} cells (i.e. dead cells) within G1+G2 was then determined using G3. Numbers on the contour plots are percentages of cells within G3. (b) Individual data points and means for six donors. Data was analysed by three-way ANOVA, and comparisons between means carried out using Fisher's LSD tests. The 5, 1 and 0.1% LSDs are depicted by the black intervals.
Figure 3: Expression of perforin and mobilisation of CD107a and CD107b in Vδ2+ T cells. (a) and (b) Flow cytometry was used to measure the expression of perforin by Vδ2+ T cells in PBMCs cultured with ZA and IL-2 for 0, 1 and 9 days. (a) Representative flow cytometry plots from one of three donors showing perforin expression in Vδ2+ T cells. Lymphocytes were gated based on FSC and SSC using G1. Note that G1 was extended at day 9 to incorporate blast cells. Vδ2+CD3+ cells within G1 were gated using G2. Percentage expression and MFI of perforin within G1+G2 was then assessed. Unfilled overlays = test, filled overlays = isotype. Numbers on the histogram plots are percentage of cells within the marker. (b) Means±SD for three donors. Test MFIs for the total G1+G2 population were divided by the isotype controls to obtain relative MFIs. Data was analysed by repeated measures one-way ANOVA, and comparisons between means carried out using Tukey’s multiple comparison tests. (c) and (d) Vδ2+ T cells were cultured with or without autologous M0, M1 or M2 Møs that had been treated with or without ZA for the last 18 hours of culture. Flow cytometry was used to measure the expression of CD107a and CD107b by Vδ2+ T cells. (c) Representative flow cytometry contour plots from one of three donors showing CD107a and CD107b expression on gated Vδ2+CD3+ cells. Lymphocytes were gated based on FSC and SSC using G1. Vδ2+CD3+ cells within G1 were gated using G2. Percentage expression of CD107a and CD107b within G1+G2 was then assessed. Quadrants were set against the Vδ2 alone controls, and separate quadrants were generated for isotype and test. Numbers are percentages of cells contained within the upper right quadrants. (d) Individual data points and means for three donors. Data was analysed by repeated measures two-way ANOVA, and comparisons between means carried out using Sidak’s multiple comparison tests. For (b) and (d), *, **, *** and **** indicate p values of <0.05, <0.01, <0.001 and <0.0001, respectively.

Figure 4: The effect of concanamycin A on Vδ2+ T cell cytotoxicity towards ZA-treated Møs. M0, M1 and M2 Møs treated with or without ZA for the last 18 hours of culture were labelled with CFSE and then cultured overnight with or without autologous Vδ2+ T cells that had been pre-treated for two hours with or without CMA (100ng/ml) or DMSO. Flow cytometry was then used to measure Zombie-NIR expression. (a) Representative flow cytometry contour plots for M0 Møs from one of five donors. The percentage of Zombie-NIRhigh cells (i.e. dead cells) within CFSE+ Møs was determined using the G1+G2+G3 gating strategy described in Fig. 2. Numbers on the plots are percentages of cells within G3. (b) Individual data points and means for five donors. Data was analysed by three-way ANOVA, and
comparisons between means carried out using Fisher’s LSD tests. The 0.1% LSD is depicted by the black interval. (c) Data in (b) was expressed as percentage inhibition. Within the +ZA data sets, the percentage of dead Mφs in the absence of Vδ2+ T cells (i.e. background cell death) was subtracted from that induced by the DMSO- and CMA-treated Vδ2+ T cells. The corrected values for Mφ cell death induced by CMA-treated Vδ2+ T cells were then expressed as a percentage of the corrected values for Mφ cell death induced by DMSO-treated Vδ2+ T cells. These values were then converted to percentage inhibition by subtracting them from 100%. Data was analysed by repeated measures one-way ANOVA, and comparisons between means carried out using Tukey’s multiple comparison tests. ** indicates a p value <0.01.
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Figure 1

(a) Flow cytometry plots showing CD64 and CD206 expression levels in different cell populations (M0, M1, M2) as indicated by the histograms for each population. The histograms display the percentage of cells (SSC) and expression levels (FSC) with G1 gates.

(b) Graphs showing the relative MFI (Mean Fluorescence Intensity) of CD64 and CD206 in M0, M1, and M2 populations. The graphs indicate significant differences as denoted by asterisks (***, ****).

(c) Graphs showing the concentration of IL-12p70 and CCL18 in M0, M1, and M2 populations. The concentration levels are measured in ng/ml and show variability across the populations.
Figure 2

(a) 

SSC

FSC

CFSE

Zombie-NIR

(b)

% dead cells

M0  M1  M2

-\(V_0^2\) -ZA  +\(V_0^2\) -ZA

-\(V_0^2\) +ZA  +\(V_0^2\) +ZA

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Figure 3

(a) Scatter plots showing flow cytometry data with G1 and G2 gates.

(b) Graphs plotting percentage of perforin-positive cells over time.

(c) Flow cytometry analysis of different conditions.

(d) Dot plot showing CD107a/b expression with significance levels.
Figure 4

(a) Flow cytometry plots showing Zombie-NIR and FSC-A glasses for different treatments.

(b) Bar graph showing % dead cells for different treatments.

(c) Graph indicating % inhibition for different treatments.
Supplementary Fig. 1: The additional Vδ1+ T cell depletion step used to increase the purity of Vδ2+ T cells from one of the donors. PBMCs were treated for 9 days with 1μM ZA and 5ng/ml IL-2. Culture medium was replaced with fresh medium containing 5ng/ml IL-2 every 2–3 days. Dead cells, non-γδ T cells and Vδ1+ cells were depleted sequentially using MACS. Flow cytometry was used to measure the percentage of Vδ2+CD3+ and Vδ1+CD3+ cells pre- and post-Vδ1+ cell depletion. Flow cytometry plots are from one donor. Dead cells and debris were excluded based on FSC and SSC, and Vδ2, Vδ1 and CD3 expression was assessed on gated cells. Numbers on the dot plots are percentages of cells contained within the quadrants. Vδ2−CD3+ cells (i.e. cells in the upper left quadrant of the middle column of dot plots) were coloured blue on the Vδ1 vs. CD3 dot plots on the right hand side.
Supplementary Fig. 2: Expansion and isolation of Vδ2+ T cells. PBMCs were treated for 9 days with 1μM ZA and 5ng/ml IL-2. Culture medium was replaced with fresh medium containing 5ng/ml IL-2 every 2–3 days. Dead cells and non-γδ T cells were then depleted sequentially using MACS. Flow cytometry was used to measure the percentage of Vδ2+CD3+ cells at day 0 and 9 pre and post depletion of dead cells and non-γδ T cells. Representative flow cytometry dot plots from one of four donors are shown. Dead cells and debris were excluded based on FSC and SSC, and Vδ2 and CD3 expression was assessed on gated cells. Numbers on the dot plots are percentages of cells contained within the upper right quadrants.
Supplementary Fig. 3: Preliminary optimisation experiments used to determine the concentration of ZA and E:T ratio for cytotoxicity assays. CD14+ cells were cultured for 2 hours in serum-free medium and then cultured for 10 days in 10% FBS medium. 25ng/ml IFN-γ was added for the last 48 hours, and 100ng/ml LPS with or without 1 or 10μM ZA was added for the last 18 hours. (a) Day 10 MΦs were washed twice in PBS and then cultured for 20 minutes in PBS containing 1μM CFSE. CFSE+ MΦs were washed three times in complete medium and cultured for 5 hours with or without autologous Vδ2+ T cells (generated as described in the materials and methods) at E:T ratios of 2:1, 5:1 and 10:1. Cells were then stained with Zombie-NIR and the percentage of Zombie NIR+/cells within CFSE+ cells determined by flow cytometry using the gating strategy described in Fig. 2. (b) Day 10 MΦs were washed three times in complete medium and cultured for 5 hours with or without autologous Vδ2+ T cells (generated as described in the materials and methods) at an E:T ratio of 2:1. Expression of CD107a and CD107b on Vδ2+ T cells was then measured by flow cytometry as described in the materials and methods using the gating strategy described in Fig. 3. For (a) and (b), means±SD for three donors are shown and the E:T ratios were based on the number of monocytes seeded at the start of MΦ differentiation. Results show Vδ2+ T cell-mediated killing of MΦs at the E:T ratio of 2:1 and the 10μM concentration of ZA.
Supplementary Fig. 4: The effect of concanamycin A on the viability of Vδ2+ T cells. Different gates were applied to the data set shown in Fig. 4. (a) Representative flow cytometry plots from one of five donors showing the gating strategy used. CFSE− cells (i.e. Vδ2+ T cells) were gated using G1. The percentage of Zombie-NIRlow cells (i.e. viable cells) within G1 was then determined using G2. (b) Means±SD for five donors.