In Vivo PET Tracking of 89Zr-Labeled Vγ9Vδ2 T Cells to Mouse Xenograft Breast Tumors Activated with Liposomal Alendronate

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Gammadelta T (γδ-T) cells are strong candidates for adoptive immunotherapy in oncology due to their cytotoxicity, ease of expansion, and favorable safety profile. The development of γδ-T cell therapies would benefit from non-invasive cell-tracking methods and increased targeting to tumor sites. Here we report the use of [89Zr]Zr(oxinate)4 to track Vγ9Vδ2 T cells in vivo by positron emission tomography (PET). In vitro, we showed that 89Zr-labeled Vγ9Vδ2 T cells retained their viability, proliferative capacity, and anti-cancer cytotoxicity with minimal DNA damage for amounts of 89Zr ≤ 20 mBq/cell. Using a mouse xenograft model of human breast cancer, 89Zr-labeled γδ-T cells were tracked by PET imaging over 1 week. To increase tumor antigen expression, the mice were pre-treated with PEGylated liposomal alendronate. Liposomal alendronate, but not placebo liposomes or non-liposomal alendronate, significantly increased the 89Zr signal in the tumors, suggesting increased homing of γδ-T cells to the tumors. γδ-T cell trafficking to tumors occurred within 48 hr of administration. The presence of γδ-T cells in tumors, liver, and spleen was confirmed by histology. Our results demonstrate the suitability of [89Zr]Zr(oxinate)4 as a cell-labeling agent for therapeutic T cells and the potential benefits of liposomal bisphosphonate treatment before γδ-T cell administration.

INTRODUCTION
Adoptive transfer of therapeutic T cells is a growing field in immunoncology, with spectacular clinical results against melanoma and hematological cancers.1-3 Gammadelta-T (γδ-T) cell therapy is one type of T cell therapy being explored, with recent data showing intra-tumoral γδ-T cells are the single most favorable prognostic immune cell infiltrate.4 γδ-T cells perform roles belonging to both adaptive and innate immunity, playing a significant role in anti-infectious and anti-tumor immune surveillance.5 Activated γδ-T cells are highly cytotoxic, enhance the function of other immune cells, and act as antigen-presenting cells.6 In humans, the Vγ9Vδ2 subtype of γδ-T cells represents 1%–5% of circulating CD3+ T cells.7 Their potent cytotoxicity and high proliferative capacity have made them candidates of choice for cancer immunotherapy.7

The unique activation of Vγ9Vδ2 cells by phosphoantigens such as isopentenyl pyrophosphate (IPP)8 allows them to discriminate between normal and metabolically disordered cells based on IPP expression levels.9 The activation and targeting of γδ-T cells to tumor tissue could, therefore, be improved by selectively increasing the presentation of phosphoantigens in cancer cells, for example, by using liposome- or nanocarrier-based formulations of aminobisphosphonate drugs (NBPs).10 NBPs (e.g., pamidronate, alendronate, and zoledronate),11 which increase the expression of IPP in target cells by inhibiting farnesyl diphosphate synthase, are hydrophilic molecules that accumulate in bone, but not in other tissues, and are rapidly cleared from the circulation. Encapsulating alendronate in liposomes has been shown to increase the therapeutic efficacy of γδ-T cells in preclinical models.12,13 Clinical studies of γδ-T cell immunotherapy have shown a good safety profile and efficacy comparable to second-line anticancer therapies, but they have also highlighted the need for improvements.14,15 Unknown aspects of adoptive γδ-T cell therapy include their in vivo distribution and kinetics of arrival at the tumor site. Whole-body imaging is highly useful in this context by enabling in vivo tracking of administered cells. Many techniques exist for non-invasive cell tracking;16-18 however, only nuclear imaging, and particularly positron emission tomography (PET), provides sensitive and quantitative, whole-body information with adequate spatiotemporal resolution. Hence, methods to radiolabel and track therapeutic cells using positron-emitting radionuclides are likely to become important tools for cell immunotherapy.19

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PET tracking of T cells has been performed with radiolabeled antibodies, antibody fragments, or lipophilic small molecules\textsuperscript{20,21} and by reporter-gene imaging.\textsuperscript{22} When genetic engineering is not required, e.g., for gd-T cells, a clinically applicable alternative to reporter-gene imaging is direct cell labeling with PET radionuclides. Immune cells have long been imaged clinically by single-photon emission computed tomography (SPECT) in this manner, for example, using \([111\text{In}]\text{In(oxinate)}\)\textsuperscript{3} and \([99m\text{Tc}]\text{Tc-exametazime}.\textsuperscript{19}\) In this regard, the clinically approved 8-hydroxyquinoline (oxine) has been recently shown to be an excellent ionophore for cell labeling with \(89\text{Zr} (t_{1/2} = 78.4 \text{ hr}, \beta^+ = 22.3\%).\textsuperscript{23–25}\) However, to the best of our knowledge, no study has evaluated its use for tracking gd-T cells.

Here we report the first use of \([89\text{Zr}]\text{Zr(oxinate)}_4\) for \textit{in vitro} radiolabeling and \textit{in vivo} tracking of human gd-T cells, including the effects of radiolabeling on gd-T cell functionality, proliferation, and DNA integrity. We applied this strategy in a xenograft model of breast cancer with an engineered cancer cell line that allows multimodal imaging to track tumor cells. A liposomal aminobisphosphonate was administered to increase T cell trafficking to the tumor.

**RESULTS**

**Radiotracer Synthesis and gd-T Cell Radiolabeling**

\(\text{(A) } [89\text{Zr}]\text{Zr(oxinate)}_4\text{ synthesis. (B) Labeling efficiencies of } \gamma\delta\text{-T cells incubated with } [89\text{Zr}]-based tracers (63.2 ± 7.9 \text{ mBq/cell}) \text{ 20 min at RT. Mean of } N = 3–4\text{ individual experiments (unpaired t test). (C) } [89\text{Zr}]\text{ retention by } \gamma\delta\text{-T cells over 7 days after labeling with } [89\text{Zr}]\text{Zr(oxinate)}_4\text{ (average incorporated activity: } 34.3 ± 6.0 \text{ mBq/cell}). \text{ Mean ± SEM of triplicate measures for } 3\text{ cell batches.}\)**

To study long-term tracer retention, radiolabeled gd-T cells (25–40 mBq/cell) were cultured at 0.83 $\times$ 10\(^6\) cells/mL. After 24 hr, the percentage of cell-associated \(89\text{Zr}\) was 72.9 ± 6.8% of the original activity, and 42.4 ± 12.6% after 1 week (\(N = 3\); Figure 1C).

**In Vitro Assays of \(89\text{Zr}\)-Radiolabeled gd-T Cells**

The purity of \textit{in vitro}-expanded gd-T cells plateaued 13–15 days post-isolation (Figure S3), at which point they were radiolabeled. Cells labeled with 6–20 mBq/cell proliferated similarly to unlabeled cells (\(p < 0.05\); Figure 2A), while cells labeled with more than 50 mBq/cell ceased to proliferate \textit{in vitro}, indicating a dose-dependent effect of \(89\text{Zr}\) on gd-T cell proliferation. A similar dose dependency was observed on gd-T cell death (Figure 2B) and DNA damage, evaluated by the formation of H2AX foci\textsuperscript{26} 1 hr after labeling (Figures 2C and 2D).

To evaluate the cytotoxic ability of radiolabeled gd-T cells, we quantified the survival of MDA-MB-231.hNIS-GFP cancer cell monolayers. gd-T cells labeled with up to 600 mBq/cell showed no significant difference in cancer cell killing compared to unlabeled gd-T cells (Figure 2E). As a control, adding \(89\text{Zr}\) up to 3 Bq/cancer cell in the medium was not toxic to cancer cells in the absence of gd-T cells. Even in 30-fold excess, gd-T cells showed no toxicity toward cancer cells in the absence of aminobisphosphonate (Figure S4).

**In Vivo PET Tracking of \(89\text{Zr}\)-Radiolabeled gd-T Cells**

\(89\text{Zr}\)-radiolabeled gd-T cells were administered intravenously in a mouse xenograft model of breast cancer followed by PET imaging...
at 1 hr, 48 hr, and 7 days after injection. We imaged the hNIS-expressing cancer cells by SPECT using $^{99m}$TcO$_4$ [27]. We also evaluated the effect of PLA on gd-T cell homing to tumor sites. The study schedule is provided in Figure 3A.

The PLA dosing schedule was established using $^{111}$In-labeled PLA, showing significant PLA tumor accumulation within 24–72 hr of administration (Figure 3B; Table S1). The experimental group (PLA treated) received radiolabeled gd-T cells + PLA (5 mg/kg alendronate).
Control groups (non-PLA treated) received radiolabeled γδ-T cells with placebo liposomes, non-liposomal alendronate, or saline. An additional control group received γδ-T cells killed by freeze-thawing to compare bio-distributions of viable and non-viable cells.

SPECT showed uptake of $^{99m}$TcO$_4^-$ in tumors and endogenous NIS-expressing organs (thyroid, salivary, and lacrimal glands and stomach; Figure 3C). At 1 hr after intravenous administration of $^{89}$Zr-radiolabeled γδ-T cells, PET revealed high amounts of radioactivity in the lungs in all groups, with signal also observed in the liver and spleen (Figures 3C and 3D). There was significantly higher uptake in the liver in the ALD group versus the PLA group. At tumor sites, the $^{89}$Zr signal was close to background (Figure S5). After 48 hr, $^{89}$Zr activity increased in the liver, spleen, and bones in all groups and decreased in the lungs. Uptake of $^{89}$Zr was observed at the tumor site only in the PLA group (Figure S5), suggesting the presence of radiolabeled γδ-T cells. Importantly, this was significantly higher in PLA-treated animals compared to control animals treated with non-liposomal alendronate (Figure 3D).

Enlarged tumor views showed heterogeneity in tumor tissue, with live tissue, expressing a functional hNIS protein$^{18,27}$ and represented by a donut of $^{99m}$Tc signal surrounding a core of non-viable tumor cells (Figure 3E). $^{89}$Zr signal in tumors was heterogeneous, with some co-localizing with $^{99m}$Tc at the edges and foci of $^{89}$Zr signal inside the tumor. After 7 days, $^{89}$Zr activity remained high in the liver; increased in the spleen, bones, and kidneys; and was indistinguishable from background in tumors. Uptake values are provided in Table S2. Compared to other treatment groups, PET images of killed γδ-T cells showed a higher accumulation in the liver immediately after injection and increased uptake of $^{89}$Zr in the kidneys at later time points (Figure S6).

**Ex Vivo Bio-distribution of $^{89}$Zr-Radiolabeled γδ-T Cells**

Ex vivo γ-counting 7 days post-administration of radiolabeled cells revealed a high concentration of $^{89}$Zr in the spleen (153.5% ± 88.8% injected dose [ID]/g averaged across all groups, N = 24) and liver (58.1% ± 10.6% ID/g, N = 24) in all groups, followed by lung and bone tissue (Figure 4A). Uptake of $^{89}$Zr in tumors from PLA-treated groups (2.1% ± 0.8% ID/g) was significantly higher than in non-PLA groups (1.2% ± 0.3% ID/g; Figure 4B), suggesting higher γδ-T cell numbers in PLA-treated tumors. Bone uptake of $^{89}$Zr in PLA-treated groups (6.5% ± 0.8% ID/g, N = 9) was significantly lower than in other groups (10.0% ± 1.1% ID/g, N = 12; p = 0.0238). Uptake in kidneys was significantly higher with killed γδ-T cells than in other treatment groups (Table S3). Uptake in other organs showed no major differences between treatment groups.

Tumor section autoradiographs showed a strong signal originating from hNIS-accumulated $^{99m}$TcO$_4^-$. Autoradiography was repeated after 4 days to allow for the decay of $^{99m}$Tc and the capture $^{89}$Zr signal.
Sections from PLA-treated animals showed increased $^{89}$Zr signal compared to non-PLA-treated animals. The $^{89}$Zr signal was higher in the tumor periphery, whereas the $^{99m}$Tc signal was uniformly distributed (Figure 4C). γδ-T cell presence in tumors was demonstrated by immunohistochemistry. Human CD3-positive cells (>95% γδ-T cell receptor [TCR]$^+$ at the time of administration; Figure S3) were visible in tumors 48 hr and 7 days after injection, both in the periphery and deeper regions (Figures 5A–5C; Figure S7). These cells were also visible in the spleen and liver after 7 days, but not in kidney sections (Figure 5D) or in control tissues of mice not administered γδ-T cells (Figure S8).

DISCUSSION

$[^{89}\text{Zr}]\text{Zr(oxinate)}_4$ synthesis has been reported previously by our group and others. The temperature-independent labeling efficiency of γδ-T cells with $[^{89}\text{Zr}]\text{Zr(oxinate)}_4$ suggests this is a passive process, in line with results from Sato et al. Sufficient radiotracer retention within cells is important to ensure that the imaging signal reflects labeled cells rather than free radiotracer bio-distribution. We observed an efflux of approximately half of the incorporated $^{89}$Zr over 1 week in vitro, which we believe does not interfere with in vivo imaging within this time frame. Uptake of $^{89}$Zr in the bone can be used to estimate the amount of tracer that leaked from the cells. Retention of $[^{89}\text{Zr}]\text{Zr(oxinate)}_4$ is dependent on cell type, and our results are comparable to those observed with dendritic, bone marrow, and chimeric antigen receptor (CAR)-T cells.

Comparable levels of tracer efflux have been observed from lymphocytes labeled with $[^{111}\text{In}]\text{In(oxinate)}_3$, the current gold standard for cell tracking by nuclear imaging.

A radiotracer for cell tracking must not significantly alter the phenotype, survival, proliferation capacity, and functionality of labeled cells. We demonstrated that the effects of $[^{89}\text{Zr}]\text{Zr(oxinate)}_4$ on γδ-T cell survival, proliferation capacity, and DNA damage were kept minimal for doses up to 20 mBq/cell but were significant at doses ≥50 mBq/cell. The cytotoxicity of radiolabeled γδ-T cells...
against the same tumor cells used for in vivo experiments was not affected by amounts of \([^{89}\text{Zr}]\text{Zr(oxinate)}_4\) of up to 600 mBq/cell, at least within 48 hr of radiolabeling. Cancer cell death was due to the combination of bisphosphonate treatment and γδ-T cells and not to the presence of \(^{89}\text{Zr}\). Preserved cytotoxicity after radiolabeling, also recently observed in CAR-T cells by Weist et al.,\(^{25}\) is encouraging for the use of \([^{89}\text{Zr}]\text{Zr(oxinate)}_4\) as a T cell-tracking agent. However, the therapeutic efficacy of γδ-T cells presumably also relies on their in vivo proliferation ability; hence, we suggest that radiolabeling γδ-T cells with \([^{89}\text{Zr}]\text{Zr(oxinate)}_4\) should ideally not exceed 20 mBq/cell. This could lead to sensitivity issues on conventional PET scanners. Indeed, our experiments show that ex vivo gamma-counting tumors could reveal amounts of \(^{89}\text{Zr}\) indistinguishable from background in our PET imaging system at day 7. Assuming that a human cell-tracking study would require 37 MBq \(^{89}\text{Zr}\)\(^{23}\) and \(^{99}\text{TcO}_4^-\) to visualize hNIS-expressing tumors by SPECT, the in vivo distribution of \(^{89}\text{Zr}\)-labeled γδ-T cells over time was similar to that observed in studies of adaptively transferred γδ-T cells\(^{35,36}\) and other T cells.\(^{23,27,38}\) \(^{89}\text{Zr}\) uptake was significantly increased in PLA-treated tumors, suggesting that PLA increases homing of these cells to the tumor site. Accumulation of γδ-T cells at the tumor site 48 hr after administration was also observed by others.\(^{35}\) Uptake values for the spleen and tumor determined by image-based quantification are lower than those determined by ex vivo bio-distribution. This can be explained by the small size of this organ and significant partial volume effect (spleen) and the liquid or necrotic tumor core that leaked upon dissection.

For in vivo studies, a xenograft model of human breast cancer in immunocompromised mice\(^{2,8}\) was chosen, as mice do not possess a subset of T cells functionally equivalent to human \(V_{γ7}\)γδT cells.\(^{34}\) We tracked γδ-T cells radiolabeled with \([^{89}\text{Zr}]\text{Zr(oxinate)}_4\) (30–300 mBq/cell) by PET 1 hr, 48 hr, and 7 days after intravenous injection. We simultaneously used \(^{99}\text{TcO}_4^-\) to visualize hNIS-expressing tumors by SPECT. The in vivo distribution of \(^{89}\text{Zr}\)-labeled γδ-T cells over time was similar to that observed in studies of adaptively transferred γδ-T cells\(^{35,36}\) and other T cells.\(^{23,27,38}\) \(^{89}\text{Zr}\) uptake was significantly increased in PLA-treated tumors, suggesting that PLA increases homing of these cells to the tumor site. Accumulation of γδ-T cells at the tumor site 48 hr after administration was also observed by others.\(^{35}\) Uptake values for the spleen and tumor determined by image-based quantification are lower than those determined by ex vivo bio-distribution. This can be explained by the small size of this organ and significant partial volume effect (spleen) and the liquid or necrotic tumor core that leaked upon dissection.

For instrument sensitivity reasons, some imaging studies were performed with higher doses of \(^{89}\text{Zr}\) than recommended above. However, the distinctly different distribution pattern observed with killed γδ-T cells suggests that radiolabeling with up to 300 mBq/cell, which preserved cytotoxic functionality in vitro over 48 hr, did not impair γδ-T cell trafficking and allowed us to track live cells. Furthermore, previous studies have shown that \([^{89}\text{Zr}]\text{Zr(oxalate)}_4\)\(^{20,39}\) \([^{99}\text{Zr}]\text{Zr(oxinate)}_4\), and lysates from \([^{89}\text{Zr}]\text{Zr(oxinate)}_4\)-labeled cells\(^{23}\) have distinct distribution patterns from intact cells labeled with \([^{89}\text{Zr}]\text{Zr(oxinate)}_4\). Cell concentrations during labeling and in vitro assays were in the range of 1–5 × 10^6/ml. In comparison, using in vitro \(^{89}\text{Zr}\) retention values, cell concentrations extrapolated from PET-computed tomography (CT) images in the organs showing the strongest \(^{89}\text{Zr}\) signal (spleen, liver, and lungs) would be in the range of 0.5–5 × 10^6 cells/ml. We therefore expect the DNA damage sustained by γδ-T cells, due to both self-irradiation and crossfire, after in vivo administration to be comparable to that observed in vitro. Considering the strong affinity of the \(^{89}\text{Zr}^{4+}\) ion for bone,\(^{28}\) the relatively low bone accumulation of \(^{89}\text{Zr}\) indicates limited efflux of weakly chelated \(^{89}\text{Zr}\), and it suggests that \(^{89}\text{Zr}\) is mostly retained by γδ-T cells after injection. The lower accumulation of \(^{89}\text{Zr}\) in the bones of PLA-treated animals compared to other groups also suggests reduced efflux of \(^{89}\text{Zr}\) from γδ-T cells after PLA treatment.

Histology confirmed the presence of γδ-T cells in the tumors, spleen, and liver, using the CD3 marker.\(^{40}\) Immunohistochemistry and autoradiography suggest that γδ-T cells accumulated mostly at the periphery of the tumor. The small number of cells observed by immunohistochemistry precludes statistical comparison. Furthermore, these techniques can only image the solid portion of the tumor. PET imaging not only allowed visualization of the whole, intact tumors but additionally revealed heterogeneous distributions of \(^{89}\text{Zr}\) in tumors, which would be challenging to observe by histology. Combined with the non-invasive nature of PET imaging, this further highlights the value of using PET tracers such as \([^{89}\text{Zr}]\text{Zr(oxinate)}_4\) for cell tracking. The high uptake of \(^{89}\text{Zr}\) in the liver and spleen was mirrored by the large numbers of human CD3+ cells observed in these tissues, consistent with the bio-distribution of radiolabeled γδ-T cells. In contrast, the apparent absence of CD3+ cells in the kidneys, despite higher \(^{89}\text{Zr}\) uptake than in the tumor, and the fact that the kidney uptake of \(^{89}\text{Zr}\) was significantly higher in animals administered killed γδ-T cells than in other groups both suggest that the radioactivity detected in the kidneys corresponds to \(^{89}\text{Zr}\) progressively released from γδ-T cells in other organs. A limitation of directly labeling cells is that the radionuclide can leak out over time and be taken up by adjacent tissue. Although immunohistochemistry demonstrates the presence of the administered γδ-T cells in the tumors, this technique cannot determine whether the \(^{89}\text{Zr}\) signal originates from the γδ-T cells or from in situ-labeled bystander cells.

A critical aspect of this type of cellular immunotherapy is that the therapeutic cells must be activated at the target site and reach the tumor in sufficient numbers. γδ-T cell toxicity toward cancer cells is greatly amplified by bisphosphonates, suggesting a role for γδ-T cells in the anti-cancer properties of bisphosphonates.\(^{41}\) Here we sought to increase phosphoantigen expression in tumors by administering PLA, which delivers alendronate to the tumors in an untargeted fashion by virtue of the enhanced permeability and retention (EPR) effect.\(^{52}\) Liposomal alendronate proved safer than other bisphosphonates and effective in potentiating γδ-T cell therapy.\(^{12,43}\) We have previously shown that the tumor-to-background uptake ratio of PLA increases over time and is significant after 3 days.\(^{41}\) Here we observed that PLA administered 4 days in advance significantly increased the amount of \(^{89}\text{Zr}\) reaching the tumor within 48 hr of radiolabeled γδ-T cell administration. Our results suggest that γδ-T cells home to the tumor within 2 days and remain there for at least 5 days. This was not observed in any other treatment group, demonstrating the importance of encapsulating the aminobisphosphonate in a tumor-targeting vehicle.
Clinical imaging studies of therapeutic T cells with \(^{111}\text{In}\)ln (oxinate)_3 have been performed by radiolabeling only a fraction of the total administered T cells,\(^ {45-47}\) although evidence exists that distributing the total activity over a larger number of cells better preserves their proliferative abilities.\(^ {48}\) Our results suggest that radiolabeling the entire batch of \(\gamma\delta\)-T cells with \([^{89}\text{Zr}]\text{Zr}(\text{oxinate})_4\) might be the preferable option to avoid imaging excessively damaged cells. In two notable studies, \(\gamma\)-scintigraphy revealed T cell uptake in tumors using only 1–3 mBq \(^{111}\text{In}\) per cell.\(^ {40,49}\) Considering the increased sensitivity of PET over SPECT and expected improvements in PET technology, clinical imaging of T cell therapies using \([^{89}\text{Zr}]\text{Zr}(\text{oxinate})_4\) is a credible prospect.

Conclusions
This study demonstrates the suitability of \([^{89}\text{Zr}]\text{Zr}(\text{oxinate})_4\) as a PET tracer to track \(\gamma\delta\)-T cells in vivo, while previous work has shown the therapeutic efficacy of \(\gamma\delta\)-T cells in combination with P.\(^ {1,4,21}\) These objectives achieved, \([^{89}\text{Zr}]\text{Zr}(\text{oxinate})_4\) can now be applied to answer fundamental questions in the preclinical and clinical development of \(\gamma\delta\)-T cell therapies, e.g., whether the accumulation of \(\gamma\delta\)-T cells at the tumor site or their distribution within the tumor correlates with therapeutic efficacy. Due to numerous molecular and cellular differences, the distribution of human \(\gamma\delta\)-T cells in an immunocompromised mouse model cannot fully predict their behavior in a human host. However, the results of this proof-of-principle study can be used to design a clinical trial that will answer the question of the distribution of \(\gamma\delta\)-T cells in humans after adoptive transfer.

Our results have implications for clinical translation, and they suggest using liposomal aminobisphosphonates as adjuncts to \(\gamma\delta\)-T cell therapy. In the context of clinical protocols involving repeated infusions of \(\gamma\delta\)-T cells,\(^ {15}\) one can envisage the use of \(^{89}\text{Zr}\)-labeled cells for the first infusion, followed by PET imaging 24–72 hr later. The number of cells trafficking to the tumor sites would then be used to decide whether to pursue with additional treatment cycles. Cell radiolabeling with \([^{89}\text{Zr}]\text{Zr}(\text{oxinate})_4\) is clinically translatable without significant methodological modifications, and the high similarity of \([^{89}\text{Zr}]\text{Zr}(\text{oxinate})_4\) to the well-established \(^{111}\text{In}\)ln(oxinate)_3 should facilitate regulatory approval. Our results support that T cell labeling with \([^{89}\text{Zr}]\text{Zr}(\text{oxinate})_4\) is a realistic option for human studies and will benefit the development of cellular immunotherapy.

MATERIALS AND METHODS

Experiment Approval
Animals experiments were approved by the UK Home Office under The Animals (Scientific Procedures) Act (1986), PPL reference 7008879 (Protocol 6), with local approval from King’s College London Research Ethics Committee (KCL-REC). Experiments using human T cells received approval from KCL-REC (Study Reference HR-16/17-3746). All donors provided written, informed consent.

Reagents, Animals, and Cells
Unless otherwise indicated, reagents were purchased from Sigma-Aldrich and Merck. Female SCID/beige (CB17.Cg-Prkdc^scid^Lyst^b6^/Jr) and Nod scid gamma (NSG) (NOD.Cg-Prkdc^scid^Il2rg^m1WJf^/-SzJ) mice (18–25 g, 10–20 weeks old) were obtained from Charles River (UK). \(\gamma\delta\)-T cells were obtained as described previously,\(^ {12}\) using zoledronate (Novartis) and interleukin-2 (IL-2) (Novartis). Full details are provided in the Supplemental Materials and Methods. Population purity was assessed by flow cytometry (BD FACSCalibur), using pan-\(\gamma\delta\) TCR (IMMU510, Beckman Coulter B49175) and anti-CD3 (OKT3, BioLegend 317307) monoclonal antibodies. Data were analyzed using Flowing version (v.)2.5.1 (http://flowingsoftware.btk. fi). Only batches with \(\geq 80\%\) \(\gamma\delta\)-positive CD3\(^+\) cells were used for further experiments (\(\geq 95\%\) for \textit{in vivo} experiments). MDA-MB-231.hNIS-GFP cells\(^ {27}\) were grown in DMEM supplemented with 10% fetal bovine serum (FBS), penicillin, streptomycin, and L-glutamine (2 mM), and they were tested for mycoplasma contamination (e-Myo PCR detection kit, Bulldog Bio).

PET Tracer Synthesis
\([^{89}\text{Zr}]\text{Zr}(\text{oxinate})_4\) was synthesized as previously described.\(^ {23}\) Full details are provided in the Supplemental Materials and Methods.

Cell Labeling
\(\gamma\delta\)-T cells expanded \textit{in vitro}\(^ {12}\) were washed with PBS (Ca\(^{2+}\)/Mg\(^{2+}\)-free) and re-suspended at 5 \(\times\) 10^6/mL in PBS at RT. \([^{89}\text{Zr}]\text{Zr}(\text{oxinate})_4\) (6–600 mBq/cell) in aqueous DMSO was added to the cell suspension, keeping DMSO concentrations \(\leq0.7\%\). Neutralized \([^{89}\text{Zr}]\text{Zr}(\text{oxalate})_4\) with an equivalent amount of DMSO was used as a control. After 10–30 min of incubation, cells were pelleted and the supernatants kept aside. The cells were washed with PBS, centrifuged, and the washings combined with the previous supernatants. The cells were suspended in growth medium or PBS for further experiments. Viability was assessed using the trypsin blue dye exclusion method. Radioactivity in re-suspended cells and combined supernatants was measured in a gamma-counter. Cell-labeling efficiency (LE\([\%]\)) was calculated as follows.

\[ LE(\%) = \frac{\text{activity of cell fraction}}{\text{activity of cell fraction} + \text{activity of combined supernatants}} \]

For radiotracer retention and cell proliferation studies, radiolabeled (or vehicle-treated) \(\gamma\delta\)-T cells were cultured as described above, and they were analyzed at various time points for viability (using trypan blue), determination of cell-associated radioactivity (by \(\gamma\)-counting), and cell death (by flow cytometry using propidium iodide [PI]; Thermo Scientific). Further details are provided in the Supplemental Materials and Methods.

Cancer Cell-Killing Assay
MDA-MB-231.hNIS-GFP cells seeded in a 96-well plate at 10^4 cells/well and incubated overnight were treated with 3 \(\mu\text{M}\) zoledronate or vehicle for 24 hr. The cells were washed and the medium was replaced with \(\gamma\delta\)-T cells in growth medium. As a control for radiolabeled \(\gamma\delta\)-T cells, an equal amount of \(^{89}\text{Zr}\) in medium was added to some wells. After 48 hr, \(\gamma\delta\)-T cells were removed by washing with PBS, and cancer cell viability was evaluated using the alamarBlue assay (Thermo...
Determination of DNA Double-Strand Breaks

Radiolabeled γδ-T cells in medium were seeded onto poly-l-lysine-coated coverslips and incubated for 1 hr. After centrifugation and gentle rinsing with PBS, the cells were fixed and permeabilized with 3.7% formalin, 0.5% Triton X-100, and 0.5% IGEPAL CA-630 in PBS, then blocked with 2% BSA and 1% goat serum. γH2AX foci were detected with an anti-γH2AX (Ser139) mouse monoclonal antibody (mAb) (1:1,600; JBW301, Merck 05-636) and goat anti-mouse AF488-immunoglobulin G (IgG) (1:500; Jackson ImmunoResearch Laboratories 115-545-062). Nuclei were detected with Hoechst 33342. Images were acquired on a TCS SP5 II confocal microscope (Leica) with a 100x/1.40 HCX PL Apochromat objective (Leica) and Leica Application Suite Advanced Fluorescence (LAS-AF) control software. Ten sections (0.4-μm thickness) were imaged. At least 30 nuclei/slide were imaged (2 slides/treatment). Maximal intensity projections of z stacks were made using ImageJ v.1.51p (https://imagej.nih.gov/ij/). Nuclei and γH2AX foci were counted using CellProfiler v.2.2.0 (http://cellprofiler.org), calculating average numbers of γH2AX foci per nucleus in each image. Full details are provided in the Supplemental Materials and Methods.

Animals, Tumor Model, and Tumor Sensitization with Liposomal Alendronate

Approximately 1.5 × 10⁶ MDA-MB-231.hNIS-GFP cells were injected subcutaneously in the mammary fat pad between the fourth and fifth nipples in the left flank; tumors were grown over 3 weeks. Animals were randomly assigned to experimental groups, and investigators were not blinded to cohort allocation when assessing outcomes. Cohort sizes were chosen based on prior experience, in compliance with local regulations concerning animal experiments. Liposomal formulations were prepared at Shaare Zedek MC as previously described. Alendronate-loaded liposomes (PLA) contained 1.5–5.4 mg/mL alendronate and 36–40 μmol/mL phospholipids. Placebo liposomes contained 20–50 μmol/mL phospholipids. PLA was co-injected with placebo liposomes for a total dose of 5 mg/kg alendronate and 4 μmol phospholipids per mouse in PLA-treated animals. Placebo-treated animals received empty liposomes corresponding to 4 μmol phospholipids per mouse. Another control group received 5 mg/kg alendronate (ALD). Formulations were injected intravenously (i.v.) 4 days before the administration of radiolabeled γδ-T cells.

In Vivo PET and SPECT Imaging of γδ-T Cells, Tumors, and PLA

89Zr-radiolabeled γδ-T cells (10⁷ cells/animal in 100 μL) were injected i.v. at t = 0 hr and imaged by PET/CT within 30 min. PET/CT imaging was performed for 30–240 min (as indicated) on a nanoScan PET-CT scanner (Mediso). For tumor imaging, 100 μL 99mTcO₄⁻ (15–25 MBq) in saline was injected i.v., and SPECT-CT was performed 40 min thereafter in a NanoSPECT/CT scanner (Mediso; 1-mm collimators, 30-min scan). PET-CT and SPECT-CT were repeated at t = 48 and 168 hr. For PLA imaging by SPECT-CT, PLA was radiolabeled with [111In]In(oxinate), and administered i.v. (7 MBq 111In/mouse) to NSG mice. PET- and SPECT-CT datasets were reconstructed using a Monte Carlo-based full-3D iterative algorithm (Tera-Tomo, Mediso). Images were co-registered and analyzed using VivoQuant v.2.50 (Invicro). Regions of interest (ROIs) were delineated for PET activity quantification in specific organs. Uptake in each ROI was expressed as a percentage of injected dose per volume (% ID/mL).

Ex Vivo Bio-distribution Studies

Mice from imaging studies were used for bio-distribution studies on day 2 or 7. After culling, organs were dissected, weighed, and γ-counted together with standards prepared from a sample of injected material. The percentage of injected dose per gram (% ID/g) of tissue was calculated. Organs were cryopreserved in optimal cutting temperature (OCT) compound (VWR) for autoradiography and/or formalin fixed and paraffin embedded (FFPE) for histologic analysis.

Immunohistochemistry

Briefly, FFPE organ blocks were sliced and stained using a Discovery XT system (Ventana Medical Systems) using the DAB Map detection kit (Ventana 760-124). For pre-treatment, CC1 (Ventana 950-124) was used. Sections were stained with anti-GFP (1/1,000; Abcam ab290, UK) or anti-CD3 (LN10, Leica CD3-565-L-CE) primary antibodies, followed by biotinylated anti-rabbit or anti-mouse IgG (1/200; Dako) secondary antibodies, as appropriate. Full details are provided in the Supplemental Materials and Methods.

Statistics

Independent experiments were performed on different days with γδ-T cell batches from different donors. Data were plotted using Prism v.7.01 (GraphPad). Differences between 2 groups were evaluated by Student’s two-tailed t test. To account for repeated measurements in a same animal or cell batch and multiple treatments tested on a same cell batch, analysis was performed using 2-way repeated-measures ANOVA in GraphPad Prism or a repeated-measures Mixed Model (MM) in InVivoStat v.3.7 (http://invivostat.co.uk/), as indicated. Dunnett’s post hoc test was applied for comparisons back to a control group, or Bonferroni correction for multiple pairwise comparisons, unless otherwise specified. Exact significance values are reported in each figure.

SUPPLEMENTAL INFORMATION

Supplemental Information includes eight figures, three tables, and Supplemental Materials and Methods and can be found with this article online at https://doi.org/10.1016/j.ymtc.2018.10.006.
CONFLICTS OF INTEREST

J.M. is chief scientific officer of Leucid Bio, a company dedicated to the commercial development of CAR-T cells for solid tumors. The authors declare no other potential conflicts of interest.

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