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Adoptive Immunotherapy of Epithelial Ovarian Cancer with Vγ9Vδ2 T Cells, Potentiated by Liposomal Alendronic Acid

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Adoptive immunotherapy using γδ T cells harnesses their natural role in tumor immunosurveillance. The efficacy of this approach is enhanced by aminobisphosphonates such as zoledronic acid and alendronic acid, both of which promote the accumulation of stimulatory phosphoantigens in target cells. However, the inefficient and nonselective uptake of these agents by tumor cells compromises the effective clinical exploitation of this principle. To overcome this, we have encapsulated aminobisphosphonates within liposomes. Expanded Vγ9Vδ2 T cells from patients and healthy donors displayed similar phenotype and destroyed autologous and immortalized ovarian tumor cells, following earlier pulsing with either free or liposome-encapsulated aminobisphosphonates. However, liposomal zoledronic acid proved highly toxic to SCID Beige mice. By contrast, the maximum tolerated dose of liposomal alendronic acid was 150-fold higher, rendering it much more suitable to in vivo use. When injected into the peritoneal cavity, free and liposomal alendronic acid were both highly effective as sensitizing agents, enabling infused γδ T cells to promote the regression of established ovarian tumors by over one order of magnitude. Importantly however, liposomal alendronic acid proved markedly superior compared with free drug following i.v. delivery, exploiting the “enhanced permeability and retention effect” to render advanced tumors susceptible to γδ T cell–mediated shrinkage. Although folate targeting of liposomes enhanced the sensitization of folate receptor–α ovarian tumor cells in vitro, this did not confer further therapeutic advantage in vivo. These findings support the development of an immunotherapeutic approach for ovarian and other tumors in which adoptively infused γδ T cells are targeted using liposomal alendronic acid. The Journal of Immunology, 2014, 193: 000–000.

Worldwide, epithelial ovarian cancer (EOC) causes 140,000 deaths each year, highlighting an unmet need for more effective treatments. We set out to develop an adoptive immunotherapy for EOC that exploits the innate and multifunctional antitumor activity of γδ T cells. In primates, most circulating γδ cells express the Vγ9Vδ2 TCR, enabling their HLA-independent activation by nonpeptide phosphoantigens (PAGs) (1). The primary sources of PAGs in humans are mevalonate pathway intermediates that lie upstream of farnesyl pyrophosphate (FPP) synthetase. Activity of the mevalonate pathway (2) or its rate-limiting enzyme (3) is commonly upregulated in ovarian and other tumors. Vγ9Vδ2 T cells also engage transformed cells through a series of innate receptor systems, including NKGD2, DNM-1, Fas ligand, and TRAIL. Upon activation, these versatile T cells provide costimulation for NK cells and interact with adaptive immune mechanisms, promoting the maturation of myeloid dendritic cells and presenting Ag to αβ T cells. Furthermore, Fc receptor expression allows some Vγ9Vδ2 T cells to mediate Ab-dependent cell-mediated cytotoxicity. These attributes render Vγ9Vδ2 T cells of great interest as mediators of antitumor immunity.

Two broad approaches have been pursued to harness the therapeutic potential of Vγ9Vδ2 T cells. These cells may be activated in vivo using aminobisphosphonates (NBP) or PAGs, an approach that has yielded some success in both adjuvant and metastatic cancer settings (4). Alternatively, ex vivo expanded Vγ9Vδ2 T cells have been adoptively infused in patients with diverse cancers, including EOC (5). Although this experience has demonstrated safety and potential for clinical efficacy, responses remain suboptimal using either strategy.

NBP drugs such as zoledronic acid (ZA) and alendronic acid (AA) inhibit FPP synthetase, thereby causing increased accumulation of PAGs (6). Consequently, ovarian and other transformed cells that take up these agents are killed more effectively by Vγ9Vδ2 T cells in vitro (7, 8). However, meaningful clinical exploitation of this...
principle is hampered by the limited cell permeability and unsatisfactory pharmacokinetic profile of NBPs. These drugs are hydrophilic, have no cell-membrane transporter, engage in negligible plasma protein binding, and consequently undergo rapid renal clearance. Poor tissue accumulation occurs at all sites with the singular exception of bone, owing to their high affinity for hydroxyapatite. Encouragingly, studies in mice engrafted with human PBMCs (including γδ T cells) indicate that retarded tumor progression occurs when small amounts of NBP gain access to established xenografts (9). Consequently, a key challenge is to alter the pharmacokinetic properties of these agents so that delivery to tumor cells is selectively enhanced. To achieve this, we evaluated liposome-encapsulated NBPs because they achieve higher and more sustained circulating drug concentrations compared with free drug (10). We have also evaluated folate targeting of liposomal NBP because this may potentiate delivery to folate receptor (FR)–expressing ovarian and other tumor cells (10). We hypothesized that an optimized liposomal NBP formulation could be used to render ovarian tumors susceptible to γδ T cell–mediated regression.

Materials and Methods

Ethical approval

Blood and tumor samples were obtained under approval of the West London Research Ethics Committee (reference 08/H0707/188; EOC patients) and the South East London Research Ethics Committee 1 (reference 09/H0804/92; healthy volunteers).

Culture of primary human γδ T cells

After isolation by gradient separation with Ficoll-Paque Plus (GE Healthcare, Chalfont St. Giles, U.K.), PBMCs were cultured at a density of 3 × 10^6 cells per milliliter in RPMI 1640 (Lonza, Basel, Switzerland), 10% human AB serum (Sigma-Aldrich, Poole, U.K.), GlutaMax (Life Technologies), and antibiotic-antimycotic solution (Life Technologies, Paisley, U.K.). On the day of isolation, ZA (1 μg/ml; Zometa; Novartis, Frimley/Camberley, U.K.), IL-2 (100 U/ml; Proleukin; Novartis), and IL-15 (10 ng/ml) were added. Additional medium and cytokines were added every 2–3 d over a total culture period of 15 d.

Where indicated, γδ T cells or NK cells were purified by positive selection using an anti-TCRγδ T cell or anti-CD56 microbead kit (Miltenyi, Bisley, U.K.), according to the manufacturer’s instructions.

Tumor cell culture

All cell lines were grown in D10 medium, such as DMEM (Lonza) supplemented with 10% FBS, GlutaMax, and antibiotic-antimycotic solution.

The firefly luciferase (luc)–expressing SKOV-3-luc-D3 cell line was purchased from Caliper (PerkinElmer). The IGROV-1-luc cell line was kindly provided by Prof. Iain McNeish (Institute of Cancer Sciences, University of Glasgow, Glasgow, U.K.). Owing to silencing or loss of luc expression, this gene was reintroduced prior to the final in vivo experiment using an SFG retroviral vector in which luc was coexpressed with dsTomato red

Table I. Abs used for flow cytometry

<table>
<thead>
<tr>
<th>Target</th>
<th>Fluorochrome</th>
<th>Source</th>
<th>Clone</th>
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<tr>
<td>EpCAM</td>
<td>PE</td>
<td>Miltenyi Biotec</td>
<td>HEA-125</td>
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<tr>
<td>FR-α</td>
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<td>R&amp;D Systems Europe</td>
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<td>Goat anti-mouse IgG</td>
<td>PE</td>
<td>Dako</td>
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<td>pan γδ TCR</td>
<td>FITC</td>
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<tr>
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<td>PE</td>
<td>BD Biosciences</td>
<td>3G8 + NCAM16.2</td>
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<tr>
<td>Isotype controls</td>
<td>All</td>
<td>BD Pharmingen</td>
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</tr>
</tbody>
</table>

Cells were stained for 20 min on ice, using the indicated Abs, as recommended by the manufacturer. All are reactive with human Ags, except where indicated.

m, mouse.
fluorescent protein. Transduced cells were then flow sorted for red fluorescent protein expression to purity.

Primary EOC tumor cells were isolated from ascites by Ficoll gradient centrifugation. Solid tumors were disaggregated with the Human Tumor Dissociation Kit (Miltenyi Biotec), used with the Gentle MACS Dissociator (Miltenyi Biotec), according to the manufacturer’s instructions. In some cases, tumor cells were cultivated as monolayers in D10 medium. Alternatively, cells were placed in Mammary Epithelial Basal Medium (Lonza) supplemented with 10% FBS and 10 ng/ml epidermal growth factor (Invitrogen), and 0.4% FBS and cultured in Ultra-Low Attachment plates (Corning, distributed by Sigma-Aldrich) for 15 d.

Liposomes containing ZA were formulated with partially hydrogenated phosphatidylcholine/cholesterol/dipalmitoyl-phosphatidylglycerol (DPPG) at a molar ratio of 55/40/5. Alternatively, DPPG was replaced with di-tearoylphosphoethanolamine-N-polyethylene glycol (PEG) 2000 at the same molar ratio (10). AA (Tokyo Chemical Company, Tokyo, Japan) was encapsulated in liposomes composed of hydrogenated phosphatidylcholine/cholesterol/di-tearoylphosphoethanolamine-N-PEG2000 at a molar ratio of 55/40/5. Lipids were lyophilized and then rehydrated in buffer containing 250 mM ammonium acetate, with pH 5.5. Resuspended liposomes were processed by serial size extrusion in a high-pressure extruder device (Lipex Biomembranes) with a temperature control at 60°C through filters with pore sizes from 1000 nm to 50 nm. Nonencapsulated AA was removed by dialysis against a buffer of 5% dextrose with 15 mM histidine, pH 7.0, followed by passage over a Dowex anion exchange resin to ensure removal of any residual free AA. The liposomes were sterilized by filtration through 0.22 μm filters and stored in Vacutainer tubes (Becton Dickinson) at 4°C. A suspension of small unilamellar liposomes of ∼100 nm diameter was obtained.

Phospholipid and NBP content was determined after Folch extraction (8:4:3 chloroform/methanol/distilled H2O or sample) to separate phospholipids (recovered in lower phase) from NBP (recovered in upper phase). Folch extraction of spiked samples of phospholipid and ZA or AA confirmed the sharp separation with no detectable overlap in the lower and upper phases of these two sources of phosphorus. Samples of each phase were assayed by the Bartlett method to determine phosphorus concentration, as described previously (11). The phosphorus measured in the lower phase represents the amount of phospholipid based on the fact that 1 mol of phospholipid contains 1 mol of phosphorus. Bisphosphonate quantification was based on phosphorus content in the upper phase, where ZA or AA each contains 2 mol of phosphorus per mol of NBP. This method can be used only for liposome preparations made with non–phosphate-containing buffer.

A lipophilic conjugate of folate-PEG5000-di-tearoyl-phosphatidylethanolamine was grafted onto preformed liposomes, with either ZA or AA, at a molar ratio of 0.5% of total phospholipid, as described (10).

Cytotoxicity assays

The 4-h cytotoxicity assays were performed by flow cytometry. In brief, target cells were stained with PKH26 (Sigma-Aldrich) according to the manufacturer’s instructions and then incubated with the indicated ZA or
AA formulations for 24 h. After washing, $1 \times 10^6$ target cells and $1 \times 10^6$ ex vivo expanded γδ T cells were cocultured at 37°C/5% CO2 for 4 h and then stained with Annexin V FITC (BD Pharmingen) according to the manufacturer’s instructions. Gating strategy is shown in Fig. 2C. Alternatively, confluent 24-well plate monolayers of SKOV-3-luc D3 and IGROV-luc cells were added to indicated wells for a further 24 h. Monolayers were then fixed and stained with crystal violet. After 72 h, residual tumor monolayers were fixed and stained with crystal violet. After a further 24 h, residual tumorsphere number was evaluated (H) Mean ± SD of three separate independent experiments, performed as described in (G). (I) IFN-γ content of supernatant harvested from cultures described above (mean ± SD, n = 3). *p < 0.05, **p < 0.01, ***p < 0.001.

Flow cytometry

Abs used for flow cytometry are listed in Table I. All flow cytometric data were acquired using a FACSCalibur cytometer and analyzed using CellQuest Pro software.

IFN-γ ELISA

Supernatants were analyzed using a human IFN-γ ELISA Ready-SET-Go Kit (eBiosciences, Hatfield, U.K.), as described by the manufacturer.

Statistical analysis

For grouped analyses, datasets were analyzed with Prism software (GraphPad, version 5) using two-way ANOVA. For comparison of two groups, datasets were analyzed with Excel within Microsoft Office for Mac 2008 (Microsoft) using a two-tailed Student t test. Survival data were analyzed using the log-rank (Mantel–Cox) test (GraphPad).
FIGURE 4. FT-L-AA sensitizes ovarian tumor monolayers to destruction by γδ T cells. IGROV-1 (A, B, C, and D) were plated in a 96-well plate in triplicate wells. On day 1, cells were pulsed with a specified concentration of free AA or nil as indicated within the indicated DPPG- (AA or nil) or PEG- (B and D) based liposomes (untargeted L-AA; FT-L-AA). On day 2, ex vivo expanded γδ T cells (E:T ratio of 10:1) or nil were added. After a further 24 h, nonadherent cells were removed, and residual tumor cell viability was determined using an MTT assay. Data represent mean ± SD of three to four independent replicates. *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001.

Results

Ex vivo expansion of γδ T cells from EOC patients and healthy donors

Patients with EOC had 14,240 ± 15,215 γδ cells per milliliter of blood (mean ± SD, n = 13), which was not significantly different from healthy donors (19,916 ± 29,887, n = 21). Following ex vivo activation with ZA, cultures became enriched for γδ T cells (Fig. 1A). This enrichment was accompanied by an average expansion of these cells by 97-fold (patients) or 172-fold (healthy donors) (Fig. 1B). Expanded γδ T cells from patients and healthy donors expressed the Vγ9Vδ2 TCR (Fig. 1C) and exhibited similar immunophenotype (Fig. 1D, 1E, Table I).

L-ZA sensitizes ovarian tumor cells to destruction by γδ T cells

The ability of ZA to sensitize tumor cells to destruction by Vγ9Vδ2 T cells is well known. We investigated whether this principle operates when ZA is encapsulated within a DPPG-containing liposome (L-ZA). Liposomes were also formulated containing folic acid (FT-L-ZA) in an effort to optimize drug delivery to FR-α+ EOC tumor cells.

Cytotoxicity experiments were first performed with PKH26-labeled IGROV-1 tumor cells, which express high levels of FR/FR-α (Fig. 2A). Tumor cells were preincubated with no drug, free ZA, L-ZA, or FT-L-ZA for 24 h prior to addition of an equal number of healthy donor–derived γδ T cells (or no T cells as control) for a further 4 h. Free ZA effectively sensitized IGROV-1 tumor cells to destruction by γδ T cells at a concentration of 1 μg/ml, indicated by an increase in Annexin V+ tumor cells. Notably, a 10-fold lower concentration of the targeted FT-L-ZA preparation (but not of untargeted L-ZA or free ZA) proved equally effective (pooled data, Fig. 2B; representative experiment, Fig. 2C). Activation of γδ T cells was accompanied by release of IFN-γ, a finding that was also enhanced most potently by FT-L-ZA pretreatment (Fig. 2D).

This principle was further tested using monolayer cultures of IGROV-1 tumor cells. At 24 h after incubation of tumor cells with ZA, L-ZA, or FT-L-ZA, ex vivo expanded γδ T cells, PHA-activated PBMCs (containing ≤5% of γδ T cells and NK cells), or no T cells were added. Residual tumor cell monolayers were visualized by crystal violet staining after a further 24 h (Fig. 2E). No monolayer destruction was observed when γδ T cells, PHA-activated PBMCs, or any ZA formulation was added alone. Free ZA or L-ZA sensitized IGROV-1 tumor monolayers to complete destruction by γδ T cells at a minimum concentration of 1 μg/ml. However, FT-L-ZA sensitized monolayers to complete destruction by γδ T cells (but not PHA-activated cells) at a 10-fold lower concentration (0.1 μg/ml; Fig. 2E). A similar sensitizing advantage for FT-L-ZA was observed using SKOV-3 tumor cells, which express lower levels of FR-α (Supplemental Fig. 1).

Recently, it has been demonstrated that ZA can also activate NK cells (14). To test whether NK cells contributed to this effect, CD16+CD56+ cells were purified from PBMCs (Fig. 2F) and were tested for their ability to mediate ZA-potentiated killing of IGROV-1 tumor cells. Although NK cells exhibited some antitumor activity in this model, no enhancement by ZA (1 μg/ml) occurred (Fig. 2G).

Patient-derived γδ T cells kill autologous ovarian tumor cells, following sensitization with free or liposome-encapsulated ZA

Next, we evaluated the cytotoxic activity of patient-derived γδ T cells against autologous EOC tumor. Initially, tumor was cultured as a monolayer over the 2-wk period required to expand γδ T cells. Following presensitization with ZA, autologous γδ T cells elicited complete monolayer destruction (Fig. 3A), accompanied by release of IFN-γ (Fig. 3B). Subsequently, tumor was propa-
gated using a spheroidal culture system that may promote stem cell self-renewal (15). Resultant "tumorspheres" expressed EpCAM, consistent with their epithelial origin (Fig. 3C), and also maintained expression of FR-α (Fig. 3D). Addition of gd T cells alone did not cause destruction of autologous tumorspheres. However, if spheres were preincubated with ZA for 24 h, they were consistently destroyed by autologous gd T cells (Fig. 3E, 3F). We next evaluated liposomal formulations of ZA in this assay system. In the absence of gd T cells, no formulation exerted any toxic effect upon tumorspheres. However, if spheres were preincubated with ZA for 24 h, they were consistently destroyed by autologous gd T cells (Fig. 3E, 3F). We next evaluated liposomal formulations of ZA in this assay system. In the absence of gd T cells, no formulation exerted any toxic effect upon tumorspheres. Similarly, gd T cells produced no antitumor activity without prior treatment with ZA. When pulsed at a concentration of 0.1 μg/ml, FT-L-ZA once again proved more effective than either free ZA or L-ZA in sensitizing tumorspheres to gd T cell destruction (Fig. 3G, 3H). Tumorsphere clearance was accompanied by IFN-γ release from gd T cells, a finding that was also enhanced most efficiently by FT-L-ZA (0.1 μg/ml), compared with other formulations tested (Fig. 3I).

L-AA sensitizes ovarian tumor cells to destruction by gd T cells

To investigate generality of concept, we compared the sensitizing capacity of free AA, L-AA, and FT-L-AA. Liposomes were formulated using either DPPG or PEG. When a limiting concentration of AA was used (0.2 μg/ml), FT-L-AA consistently proved superior in its ability to sensitize both IGROV-1 (Fig. 4A,
4B) and SKOV-3 tumor cells (Fig. 4C, 4D) for destruction by ex vivo expanded γδ T cells. Activation of γδ T cells was accompanied by release of IFN-γ (data not shown). Similar findings were obtained in monolayer destruction assays (Fig. 4E, 4F).

L-ZA is ineffective as an in vivo sensitizer to adoptive immunotherapy using γδ T cells

We found that the maximum tolerated dose of L-ZA/FT-L-ZA in SCID Beige mice was 1 mg, a dose that also resulted in transient depletion of peritoneal macrophages (Supplemental Fig. 2). Because toxicity is macrophage dependent (16), the resultant macrophage depletion allowed us to administer up to 5 µg of this agent safely within 24 h. Nonetheless, we were unable to establish conditions whereby L-ZA or FT-L-ZA could effectively sensitize either established IGROV-1 or SKOV-3 tumors to γδ T cell immunotherapy (Fig. 5). By contrast, administration of 1 µg FT-L-ZA, followed by 28 µg of FT-L-AA, resulted in modest regression of advanced SKOV-3 tumors following γδ T cell infusion (Supplemental Fig. 3).

AA effectively sensitizes epithelial ovarian tumor xenografts to adoptive immunotherapy using γδ T cells

Next, we further investigated the use of free and liposome-encapsulated AA as sensitizing agents to γδ T cell immunotherapy. PEGylated rather than DPPG liposomes were used because we have recently shown that the former achieve prolonged circulation time (16). Safety testing in SCID Beige mice indicated that FT-L-AA was well tolerated at doses ≤ 150 µg (Supplemental Fig. 4A).

To test efficacy, a pilot experiment was performed in mice with established SKOV-3-luc xenografts. A cautious dosing regimen was employed whereby mice received doses of 15 + 15 µg or 15 + 30 µg, administered i.p. and separated by 24 h. Modest tumor regression ensued in many of the mice, with a suggestion of greatest sensitization with L-AA (Supplemental Fig. 4B–D).

We next escalated the dose of AA to 30 followed by 100 µg, injected i.p. and separated by 24 h. After a further 24 h, γδ T cells were injected i.p. On this occasion, pronounced and sustained tumor regression was observed in all mice that received this drug, followed by γδ T cells (Fig. 6). Both free AA and L-AA were equally effective at this dose, without any clinical evidence of toxicity. Surprisingly, however, FT-L-AA proved less potent as an in vivo sensitizing agent to γδ T cell immunotherapy.

The i.v. delivered L-AA achieves optimal tumor sensitization to γδ T cell immunotherapy

Finally, we designed a further in vivo experiment to explore whether liposome encapsulation and/or folate targeting might confer an advantage if AA was administered i.v. This study was performed in mice with the more aggressive IGROV-1-luc tumor model. PEGylated liposomal NBPs persist for ≤ 1 wk when injected i.v. in mice, whereas free drug is cleared from the circulation within 1 h (10). Consequently, we hypothesized that repeated dosing with γδ T cells would be beneficial in light of the limited in vivo longevity of these T cells in SCID Beige mice. To test this, mice received 150 µg of free AA, L-AA, or FT-L-AA i.v. After 24, 72, and 120 h, animals received 10 million γδ T cells,
injected i.p. Under these conditions, free AA was ineffective as a tumor sensitizer. By contrast, both liposomal formulations proved highly effective such that infused γδ T cells elicited the regression of this established aggressive tumor (Fig. 7A, 7B), leading to prolonged survival of mice (Fig. 7C). Once again, L-AA proved superior to FT-L-AA in mediating tumor regression, although survival advantage was similar with both liposomal formulations. Tumor response was accompanied by mild and reversible toxicity, indicated by transient weight loss (Fig. 7D) and piloerection.

Discussion
EOC exists in a dynamic interrelationship with the immune response (17). Vγ9V62 T cells are well placed to influence this because they can detect genomic, metabolic, and signaling perturbations that are characteristic of cancer (18–20). Following adoptive transfer or in vivo activation, these innate T cells delay the progression of tumor or leukemic xenografts (9, 21–27). However, regression of established malignancy has proved more difficult to achieve using these cells. In this article, we describe
Indeed, TAMs mediate 10-fold greater drug uptake of folate-binding cells. Despite extensive validation of this drug delivery system circulating numbers of gd T cells, the permeability and retention effect is likely to be further reduced. Enhanced uptake by hepatic reticuloendothelial cells (16, 34). As these cells, second, incorporating folic acid into liposomes leads to a simultaneously achieving more efficient and sustained elimination of targeted liposomes, compromising drug delivery to tumor cells while cooperating with infused T cells to mediate the rejection of (CD45RO+ CD27+) central memory cells (35). Thus, autologous T cells have a higher therapeutic index than does ZA.

When administered directly to the site of disease, both free and L-AA proved highly effective in sensitizing tumors to gd T cell–mediated shrinkage. By contrast, i.v. injected free AA was completely ineffective, a deficiency that was rectified using liposomal encapsulation. Liposomal drug delivery to tumor deposits benefits from the “enhanced permeability and retention effect,” whereby preferential extravasation of these particles occurs across hyper-permeable tumor-associated blood vessels (29). Local drug concentrations are further enhanced owing to the lack of effective lymphatic drainage within tumors, creating a reservoir of the therapeutic agent at the site of disease (30, 31). Clinical exploitation of this principle offers the promise that cytotoxic activity of gd T cells would be targeted more precisely to tumor deposits, rather than healthy tissue, following infusion into the peritoneal cavity.

We also explored the use of folate-targeted liposomes, which we hypothesized would achieve greater NBP delivery to FR−α tumor cells. Despite extensive validation of this drug delivery system in vitro, no additional tumor-sensitizing advantage was conferred in vivo. Two factors may account for this finding. First, FRs are very highly expressed on tumor-associated macrophages (TAMs). Indeed, TAMs mediate 10-fold greater drug uptake of folate-targeted liposomes than do FR−α tumor cells such as IGROV-I (32). TAMs are highly enriched in EOC and in both tumor models studied in this research (32, 33) and are of established importance in cooperating with infused T cells to mediate the rejection of EOC tumors (13). Consequently, TAMs may act as a sink for folate-targeted liposomes, compromising drug delivery to tumor cells while simultaneously achieving more efficient and sustained elimination of these cells. Second, incorporation of folic acid into liposomes leads to their accelerated systemic clearance in vivo, largely through enhanced uptake by hepatic reticuloendothelial cells (16, 34). As a result, efficiency of liposomal drug delivery by the enhanced permeability and retention effect is likely to be further reduced.

Patients with newly diagnosed EOC had somewhat lower circulating numbers of gd T cells than did healthy donors. Nonetheless, these cells could be expanded efficiently and were functionally competent, mediating the NBP-dependent destruction of EOC tumor cell lines and autologous tumorspheres. Expansion of gd T cells with a predominantly effector memory phenotype has been reported previously from patients with EOC (8), although a subset of patient samples were refractory to this approach. The expansion protocol used in this study differed in several respects and generally yielded a greater proportion of (CD45RO+ CD27+) central memory cells (35). Thus, autologous immunotherapy using patient-derived gd T cells represents one natural extension of the preclinical approach described in this article.

Because gd T cells are HLA unrestricted and do not mediate graft versus host disease, the development of allogeneic “off the shelf” therapies using such expanded cells represents an increasingly attractive alternative. Adoptive immunotherapy using haploidentical purified gd T cells has recently achieved impressive results in a small-scale clinical study in lymphodepleted patients with treatment-refractory hematologic malignancy (36). Furthermore, Cooper et al. (37) have developed an efficient K562-based feeder system that expands polyclonal gd T cells with cytolytic activity against tumor cells and derived xenografts that are naturally sensitive to these cells. Although gd T cells have modest intrinsic antitumor activity, a key advantage associated with the use of these cells is the potential to target and regulate their activity in a pharmacologically dependent manner, as demonstrated in this article. To facilitate clinical translation, we have adapted our gd T cell expansion system to include only clinical-grade materials, eliminating the need for serum supplementation, stimulatory Abs, or feeder cells. With the use of healthy donor PBMCs, this process yields an average 2057-fold expansion of gd T cells over 2 wk (A.C. Parente-Pereira and J. Maher, unpublished observations), greatly exceeding that obtained using samples from patients. Combined use with liposome-encapsulated AA warrants clinical evaluation in patients with solid tumors.

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Disclosures
The authors have no financial conflicts of interest.

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Supplemental Figure 1. Folate-targeted liposomal ZA sensitizes SKOV-3 ovarian carcinoma cells to destruction by γδ T-cells.
(A) Flow cytometry of SKOV-3 cells following incubation with pan anti-folate receptor (FR) ab or FR-α-specific ab (open histograms). Filled histograms – isotype controls. (B) PKH26-labeled SKOV-3 cells were pulsed for 24h with free ZA or the indicated DPPG-based liposomal preparations (L-ZA untargeted liposomal ZA; FT-L-ZA – folate-targeted L-ZA). Tumor cells were then co-cultivated with an equal number of healthy donor-derived γδ T-cells for 4h. Dead tumor cells were identified as PKH26+ Annexin V+ events (mean ± SD, n=3). (C) Confluent SKOV-3 monolayers grown in a 24 well plate were pulsed with the indicated ZA formulations. After 24h, 1 x 10^6 PHA-activated PBMC or ex-vivo expanded γδ T-cells or nil were added. After a further 24h, non-adherent cells were removed and residual monolayers were fixed and stained with crystal violet. Representative microscopic field are shown. Similar results were obtained in eight independent experiments (healthy donors, n=5; EOC patients, n=3). (D) Supernatants were harvested from experiments described in (C) and analyzed for IFN-γ by ELISA (mean ± SD, n=3 using cells from patients with EOC). * p≤0.05; ** p≤0.01; *** p≤0.001; NS – not significant.
Supplemental Figure 2. Safety testing of liposomal zoledronic acid.
Two SCID Beige mice per group were injected using the i.v. or i.p. routes with either 1μg (A) or 5μg (B) of L-ZA and were weighed at the indicated time points. A similar dose was re-injected weekly in surviving mice, as indicated by overhead arrows. * Indicates that animals had either died (n=2) or were culled (n=1) owing to clinically evident toxicity. Toxicity in this model results from activation of macrophages and mast cells, meaning that higher doses are tolerated in mice following macrophage depletion. (C) Two or three SCID Beige mice were injected with 1μg of the indicated formulation of free or liposomal ZA. After 24 hours, peritoneal lavage was performed and macrophages were identified in each mouse as CD45⁺ F4/80⁺ cells. Similar results were obtained in 3 independent experiments.
Supplemental Figure 3. Folate-targeted liposomal AA can sensitize advanced SKOV-3 ovarian tumor xenografts to infused γδ T-cells. Mice (n=3 per group) with 38 day established i.p. SKOV-3-luc tumor xenografts were treated i.p. with 1μg of FT-L-ZA (to deplete macrophages) followed by either FT-L-ZA (5μg - group 1) or FT-L-AA (28μg – group 2) on day 39. Group 3 mice received PBS as control. On day 40, animals in groups 1 and 2 received 10 million γδ T-cells, injected i.p. Bioluminescence imaging was performed weekly thereafter to monitor tumor status.
Supplemental Figure 4. Pharmaco-immunotherapy of EOC with free or liposomal AA, followed by γδ T-cells.

(A) Safety testing - Two SCID Beige mice per group were treated with the indicated dose of FT-L-AA. Clinical status and weight was documented at the specified time points. In a pilot efficacy study, SCID Beige mice were inoculated i.p. with SKOV-3-luc tumor cells. On days 16 and 17, animals were treated i.p. with 2 x 15μg (B) or 15 + 30μg (C) of the indicated formulation of AA followed on day 18 by 2 x 10^7 ex-vivo expanded γδ T-cells or nil by i.p. injection. Tumor status was monitored by serial BLI (mean ± SD, n=3-5). Images of individual mice before and after treatment are shown in D, maintaining the same scale throughout.