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Adoptively-transferred ex vivo expanded $\gamma\delta$ -T cells mediate in vivo antitumor activity in preclinical mouse models of breast cancer

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Abstract

In contrast to antigen-specific $\alpha\beta$ -T cells (adaptive immune system), $\gamma\delta$ -T cells can recognize and lyse malignantly transformed cells almost immediately upon encounter in a manner that does not require the recognition of tumor-specific antigens (innate immune system). Given the well-documented capacity of $\gamma\delta$ -T cells to innately kill a variety of malignant cells, efforts are now actively underway to exploit the antitumor properties of $\gamma\delta$ -T cells for clinical purposes. Here, we present for the first time preclinical in vivo mouse models of $\gamma\delta$ -T cell-based immunotherapy directed against breast cancer. These studies were explicitly designed to approximate clinical situations in which adoptively-transferred $\gamma\delta$ -T cells would be employed therapeutically against breast cancer. Using radioisotope-labeled $\gamma\delta$ -T cells, we first show that adoptively-transferred $\gamma\delta$ -T cells localize to breast tumors in a mouse model (4T1 mammary adenocarcinoma) of human breast cancer. Moreover, by using an antibody directed against the $\gamma\delta$ -T cell receptor (TCR) we determined that localization of adoptively-transferred $\gamma\delta$ -T cells to tumor is a TCR-dependant process. Additionally, biodistribution studies revealed that adoptively-transferred $\gamma\delta$ -T cells traffic differently in tumor-bearing mice compared to healthy with fewer $\gamma\delta$ -T cells localizing into the spleens of tumor-bearing mice. Finally, in both syngeneic (4T1) and xenogeneic (2Lmp) models of breast cancer, we demonstrate that adoptively-transferred $\gamma\delta$ -T cells are both effective against breast cancer and are otherwise well-tolerated by treated animals. These findings provide a strong preclinical rationale for using ex vivo expanded adoptively-transferred $\gamma\delta$ -T cells as a form of cell-based immunotherapy for the treatment of breast cancer. Additionally, these studies establish that clinically-applicable methods for radiolabeling $\gamma\delta$ -T cells allows for the tracking of adoptively-transferred $\gamma\delta$ -T cells in tumor-bearing hosts.

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Keywords

$\gamma\delta$ -T cells; Immunotherapy; Cell therapy; Innate Immunity

Introduction

Unlike $\alpha\beta$ -T cells which require the recognition of specific processed peptide antigens presented by major histocompatibility complex (MHC) class-I or class-II molecules (adaptive immunity), $\gamma\delta$ -T cells in contrast, appear to recognize and respond to a variety of stress-induced self antigens commonly displayed by cells having undergone malignant transformation [6, 12-15,18]. Thus, while incapable of recognizing tumor-specific antigens per se, $\gamma\delta$ -T cells can nonetheless recognize malignantly transformed cells – particularly malignant cells of epithelial origin – through less specific mechanisms that require no prior antigen exposure or priming (innate immunity). Consequently, $\gamma\delta$ -T cells can recognize and lyse malignantly transformed cells almost immediately upon encounter – consistent with their role as a component of the innate immune system.

Given the well-documented capacity of $\gamma\delta$ -T cells to innately kill a variety of malignant cells, efforts are now actively underway to develop and refine the means to exploit the antitumor properties of $\gamma\delta$ -T cells for clinical purposes [4,10,11,23,42]. Over a decade ago, studies documented the presence of $\gamma\delta$ -T cells among lymphocytic infiltrates within breast tumors and their principle draining lymph nodes [1,3]. More recently, our group has reported that ex vivo expanded human $\gamma\delta$ -T cells effectively killed a panel of human breast cancer cell lines in vitro, and notably, failed to lyse normal, control human fibroblasts [17]. These in vitro findings by our laboratory have since been confirmed and extended by other groups [30,41]

While the previous findings by ourselves and others have been important in establishing the theoretical potential of $\gamma\delta$ -T cell-based immunotherapy for the treatment of breast cancer, such studies have been until now, either observational or limited to in vitro studies. In this report, we present for the first time preclinical in vivo mouse models of $\gamma\delta$ -T cell-based immunotherapy directed against breast cancer. These studies were explicitly designed to approximate clinical situations in which adoptively-transferred $\gamma\delta$ -T cells would be employed therapeutically against breast cancer. Using radioisotope-labeled $\gamma\delta$ -T cells, we show that adoptively-transferred $\gamma\delta$ -T cells do indeed localize to breast tumors in a mouse model of human breast cancer. Subsequently, in both syngeneic and xenogeneic models of breast cancer, we demonstrate that adoptively-transferred $\gamma\delta$ -T cells are both effective against breast cancer and are well-tolerated. Thus, these findings provide a strong biological rationale to justify the clinical use of adoptively-transferred $\gamma\delta$ -T cells as a form of cancer immunotherapy for breast cancer.

Materials and Methods

Mice

Female BALB/c wild-type mice and BALB/c TCR $\alpha\beta$ -deficient (TCR $\alpha\beta^{-/-}$) mice were purchased from The Jackson Laboratory, and athymic mice (NCR-^{nu/nu}) were purchased from Frederick Labs (National Cancer Institute). Mice were between 7 and 12 weeks of age and were maintained in pathogen-free facilities in accordance with the guidelines of the Animal Care and Use Committee at The University of Alabama at Birmingham (Birmingham, AL).

Cell lines

BALB/3T3 normal fibroblast (H-2^d) and 4T1 mammary adenocarcinoma (H-2^d) cell lines were purchased from the American Type Culture Collection. 4T1-*luc2* was purchased from Caliper Life Sciences (<http://www.caliperls.com>). Cells were maintained as recommended by ATCC or the supplier. For xenograft studies, the human breast cancer cell line 2LMP/*Luc* was derived by transducing 2LMP cells to express firefly luciferase using the recombinant adeno-associated virus-2 transduction methods [7,32-35].

Preparation of mouse and human $\gamma\delta$ -T cells

$\gamma\delta$ -T cells used in cytotoxicity assays and immunotherapy studies were obtained from spleen cells derived from BALB/c mice lacking $\alpha\beta$ -T cells (TCR $\alpha\beta^{-/-}$) as previously described [28]. Briefly, whole spleens were resected from TCR $\alpha\beta^{-/-}$ mice, homogenized, and then subjected to density gradient centrifugation. Cells were cultured as previously described [26] and were harvested after eight days in culture and were employed as effector cells at a 10:1 effector:target ratio against 4T1 and BALB/3T3 target cell lines in a standard four hour co-culture incubation period. Human $\gamma\delta$ -T cells for xenograft studies were prepared as previously described [29].

Flow cytometry

To assess purity of $\gamma\delta$ -T cells employed in in vitro cytolytic assays and adoptive transfer studies, flow cytometry was performed using a FACSCalibur flow cytometer (BD Biosciences, San Jose, CA). The following antibodies were used for flow cytometry and were obtained from BDBiosciences: anti-CD3-APC (clone 145-2C11), anti-CD3-FITC (clone 145-2C11), anti-TCR- $\gamma\delta$ -FITC (clone GL3), anti-TCR- $\gamma\delta$ -PE (clone GL3), anti-CD16/CD32 (clone 2.4G2). Cell preparations were stained in FACS buffer (HBSS with 5% FBS). Living cells were distinguished from dead cells using propidium iodide (PI) uptake as previously described [16,29].

In vitro cytotoxicity assay

The cytotoxicity of ex vivo expanded BALB/c-derived $\gamma\delta$ -T cells against 4T1 and BALB/3T3 cell lines was measured using the standard ⁵¹Cr release assay as we have previously described [29] or using the CytoTox96 Non-Radioactive Cytotoxicity Assay (Promega, Madison Wisconsin), an assay which quantitatively measures lactate dehydrogenase (LDH), a stable cytosolic enzyme released upon cell lysis. Both 4T1 and 4T1-*luc2* cells were determined to be equivalently sensitive to $\gamma\delta$ -T cell killing in vitro (data not shown).

Biodistribution studies using ¹¹¹In-labeled $\gamma\delta$ -T cells

Eight-week old female BALB/c mice were sham-injected with saline, or received 4×10⁴ 4T1 mammary adenocarcinoma cells in the mammary fat pad. After 20 days, mice were injected intravenously with 5×10⁶ BALB/c-derived ¹¹¹In-labeled $\gamma\delta$ -T cells, labeled with the standard nuclear pharmacy ¹¹¹In-oxine method at 5 pCi/cell (Birmingham Nuclear Pharmacy, Birmingham, AL) as previously described [8,22]. For biodistribution experiments, animals were sacrificed at 48 hours and tissues were counted with a gamma scintillation counter with results expressed as % of injected dose (ID)/g of tissue. To inhibit $\gamma\delta$ -T cell localization to tumor, $\gamma\delta$ -T cells were first pretreated (prior to injection) with 10 μ g/ml anti- $\gamma\delta$ -TCR monoclonal antibody clone GL3 for 15 minutes on ice. The GL3 antibody has been shown to downregulate the $\gamma\delta$ -TCR, which acts to functionally impair $\gamma\delta$ -T cells [20,24].

In Vivo SPECT/CT Imaging Study

Animals were maintained with isoflurane gas anesthesia for all studies, and monitored continuously to allow the lowest dose (typically 1.5-2.0%) to prevent movement. Imaging

studies were conducted using X-SPECT, a SPECT/CT dual-modality imager (Gamma Medica-Ideas, Northridge, CA) as described previously [9]. The SPECT/CT images were acquired at 48 hours following injection of ^{111}In -labeled $\gamma\delta$ -T cells. Radiation decay of ^{111}In was considered, and the same color scale was applied to all SPECT images.

Bioluminescence studies

To assess tumor response in the syngeneic mouse tumor model (i.e., BALB/c mice harboring 4T1 tumors treated with BALB/c $\gamma\delta$ -T cells), tumors were first established in otherwise healthy mice. In these studies, 4×10^4 4T1-*luc2* cells were introduced into the mammary fat pad. After two days, mice were imaged and matched into pairs based upon equivalent tumor burden and randomly assigned to a treated or a control group. Treated mice received three doses of syngeneic $\gamma\delta$ -T cells (5×10^6 per dose) delivered by intravenous injection at 2, 5 and 9 days after establishment of tumors. For xenograft studies, human 2LMP/*Luc* cells (1×10^6 cells) were introduced subcutaneously into nude mice. By convention, the day of tumor implantation is designated day 0. On day 6, animals were imaged to determine the amount of detectable tumor, matched into pairs based upon equivalent tumor burden, then randomly assigned to a treated or a control group. Treated animals received 20×10^6 human $\gamma\delta$ -T cells intravenously on days 6, 9, 13, 16, 20 and 23. Untreated animals received sham-injections. Bioluminescence images in both syngeneic and xenograft studies were obtained using the IVIS 100 imaging system (Xenogen) as has been described in detail elsewhere [21]. Briefly, each mouse was injected with luciferin (2.5 mg) and imaged after 10 min in a 37 °C chamber according to the following parameters: 25-cm axial field of view, 1-30 second exposure, photographic binning of 4 or 8, and an F-stop of 1. Identically sized regions of interest were manually drawn to surround all tumor cells, and the light emitted from the tumor cells within the region of interest was measured using Living Image 3.1 software.

Statistical analysis

In $\gamma\delta$ -T cell biodistribution studies, the mean percentage of injected dose per gram in various tissues was compared in healthy and 4T1-bearing mice using the Student's *t* test. In both the syngeneic and xenograft immunotherapy studies, the mean tumor burden was compared in treated and untreated mice using the Student's *t* test.

Results

Syngeneic $\gamma\delta$ -T cells are cytolytic in vitro against 4T1 mammary adenocarcinoma cells but not against non-malignant (control) fibroblasts

The 4T1 mammary adenocarcinoma cell line was derived from a spontaneously arising BALB/c mammary tumor and is commonly used in studies intended to approximate human breast cancer [2,40]. As an initial step in the development of our syngeneic $\gamma\delta$ -T cell immunotherapy model for breast cancer, we determined that syngeneic BALB/c cells could indeed kill 4T1 mammary adenocarcinoma cells in vitro. Importantly, we concurrently determined that normal syngeneic fibroblasts are not killed by these same $\gamma\delta$ -T cells, indicating that $\gamma\delta$ -T cells are capable of distinguishing malignant from non-malignant (i.e., normal) tissues. As shown in Figure 1, ex vivo expanded BALB/c-derived $\gamma\delta$ -T cells (H-2^{d}) kill syngeneic 4T1 (H-2^{d}) cells but mediate reduced cytotoxicity against syngeneic non-malignant BALB/3T3 (H-2^{d}) cells. This inability of activated (ex vivo expanded) $\gamma\delta$ -T cells to kill non-malignant fibroblasts is important as this would suggest that in a clinical setting, ex vivo expanded, patient-derived (i.e., autologous) $\gamma\delta$ -T cells will similarly not interact with normal tissues upon adoptive transfer. Together, these findings support our view that the 4T1 model is a feasible platform in which to continue the study of how ex vivo expanded $\gamma\delta$ -T cells might be used in the treatment of breast cancer.

Adoptively-transferred $\gamma\delta$ -T cells localize to tumors

We next designed a series of studies to determine the extent to which adoptively-transferred $\gamma\delta$ -T cells could localize within primary mammary tumors. Previously, using a syngeneic mouse model of prostate cancer, we showed that $\gamma\delta$ -T cells expressing green fluorescence protein (GFP) readily localized into established tumors [26]. However, in a clinical setting, it is unlikely that an approach employing GFP-expressing $\gamma\delta$ -T cells would be feasible. Accordingly, in this present study, we employed radiolabeled (^{111}In) $\gamma\delta$ -T cells, noting that ^{111}In -labeling is a clinically applicable method which can be used to assess the trafficking patterns of adoptively-transferred T cells in patients [31]. Using SPECT/CT imaging, Figure 2 shows that ^{111}In -labeled $\gamma\delta$ -T cells do indeed localize to 4T1 mammary fat pad tumors. Importantly, when $\gamma\delta$ -T cells were first pretreated (prior to injection) with an anti- $\gamma\delta$ -TCR monoclonal antibody which is known to downregulate the $\gamma\delta$ -TCR [20,24], a marked decrease in localization of $\gamma\delta$ -T cells to tumor was observed (Figure 2). To support these SPECT/CT findings, biodistribution studies were also performed in which mice were treated with radiolabeled $\gamma\delta$ -T cells, which were either pretreated (blocked) with the anti- $\gamma\delta$ -TCR antibody GL3 antibody, or left untreated (unblocked). Tumors were then resected from these mice. As a quantitative measure of $\gamma\delta$ -T cells localizing to tumors, radioactivity accumulating within tumor tissues was expressed (by convention) as the percent of injected radiation dose found per gram of tumor tissue. When comparing mice treated with either blocked or unblocked $\gamma\delta$ -T cells, a 34.6% reduction in $\gamma\delta$ -T cell localization to tumor was observed (2.6% of injected dose per gram of tissue when unblocked; 1.7% injected dose per gram of tissue when blocked; $p = 0.004$; data not shown). We interpret this to indicate that for adoptively-transferred $\gamma\delta$ -T cells, either trafficking or the functional accumulation within tumor is to some degree dependent on the expression of a functional $\gamma\delta$ -TCR.

$\gamma\delta$ -T cell trafficking patterns differ between healthy and tumor bearing mice

The demonstration that adoptively-transferred $\gamma\delta$ -T cells localize to mammary adenocarcinoma tumors is central to this present study. However, we were also able to assess the overall systemic tissue biodistribution of adoptively-transferred ^{111}In -labeled $\gamma\delta$ -T cells. In these studies, both healthy BALB/c mice and 4T1-bearing BALB/c mice were injected with 5×10^6 ^{111}In -labeled- $\gamma\delta$ T cells and compared. After 48 h, mice were sacrificed and tissues were removed to assess the distribution of adoptively-transferred $\gamma\delta$ -T cells within separate tissues — which by convention, is expressed as a percentage of injected radiation dose per gram of target tissue [5]. Figure 3 compares the tissue biodistribution of adoptively-transferred $\gamma\delta$ -T cells in healthy BALB/c and tumor-bearing BALB/c mice. Curiously, when comparing healthy mice and 4T1-bearing mice, $\gamma\delta$ -T cell biodistribution was similar in all tissues with the exception of the spleen where tumor-bearing mice had a significantly lower accumulation of $\gamma\delta$ -T cells in the spleen compared to healthy mice (15.3% in 4T1-bearing mice; 37% in healthy mice; $p = 0.003$).

Adoptively-transferred syngeneic mouse $\gamma\delta$ -T cells moderate the growth of mammary adenocarcinoma tumors

Having established above that $\gamma\delta$ -T cells do indeed localize to tumor, we next assessed to what extent adoptively-transferred $\gamma\delta$ -T cells could moderate mammary tumor progression. For these studies, tumor was first established by injection of a luciferase-expressing 4T1 cell line (4T1-*Luc2*) into the mammary fat pads of otherwise healthy wild type female BALB/c mice. Figure 4 shows that tumor-bearing mice treated with $\gamma\delta$ -T cells had a significant reduction in mammary tumor growth compared to untreated tumor-bearing mice. Importantly, during the course of these studies no untoward side effects were observed in mice treated with $\gamma\delta$ -T cells.

Adoptive transfer of human $\gamma\delta$ -T cells can moderate the growth of xenogeneic breast tumors

To further assess the therapeutic potential of administering $\gamma\delta$ -T cells for the treatment of breast cancer, we used a human xenograft model of breast cancer. Here, we employed the 2Lmp/*Luc* cell line, a subclone of the human breast cancer cell line MDA-MB-231 that was engineered to express luciferase [7,32-35]. As reported, human $\gamma\delta$ -T cells expanded ex vivo have been shown to reduce tumor burden and improve survival when injected into SCID mice harboring melanoma or pancreatic cancer cells [19]. Consistent with this report, as shown in Figure 5, adoptively-transferred human $\gamma\delta$ -T cells were clearly able to control the growth of human 2Lmp/*Luc* cells first xenografted into athymic (nude) mice. Similar to the above syngeneic studies, no untoward side effects were observed in mice treated with human $\gamma\delta$ -T cells.

Discussion

To date, most immune cell-based cancer immunotherapy strategies have focused on the stimulation of anti-tumor properties of the adaptive immune system, which are typically directed against tumor-specific or tumor-associated antigens. In contrast, $\gamma\delta$ -T cells can recognize generic antigens commonly expressed by stressed cells such as malignantly-transformed cells. Indeed, cancerous cells can display a number of stress-induced antigens which while neither tumor-specific nor tumor-derived, can nonetheless serve as recognition determinants for human and mouse $\gamma\delta$ -T cells [6,12-15,18]. Given the recognized capacity of $\gamma\delta$ -T cells to directly recognize and kill malignant cells both *in vitro* and *in vivo* efforts are now actively underway to develop and refine the means to exploit the antitumor properties of $\gamma\delta$ -T cells for clinical purposes [4,10,11,17,²³,28,36-38,42].

Although it remains to be determined how $\gamma\delta$ -T cells might best be utilized clinically for the treatment of malignancies, two specific approaches are being developed in this regard. One approach relies upon the activation or expansion of the endogenous $\gamma\delta$ -T cells within patients through the clinical administration of pharmacologic agents capable of stimulating human $\gamma\delta$ -T cells. This includes the use of the aminobisphosphonate drugs, typically administered in conjunction with interleukin (IL)-2 [10,11,25,39,42].

Alternatively, the innate antitumor properties of $\gamma\delta$ -T cells may also be exploited through the adoptive transfer of $\gamma\delta$ -T cells first expanded ex vivo, then subsequently reinfused into tumor-bearing patients. This latter approach — approximated by these current animal studies — while technically more involved, is nevertheless now entirely feasible in the clinical setting as advances by our group as well as others have made possible the large-scale expansion of human $\gamma\delta$ -T cells which retain antitumor activity against a variety of human tumor cell lines in vitro [4,17,28,29]

With this in mind, our current report has several limited, but nevertheless important, objectives. Foremost, this work establishes for the first time that ex vivo expanded adoptively-transferred $\gamma\delta$ -T cells can indeed limit the in vivo progression of disease in animal models of breast cancer. Although previously shown in other disease models [19] here for the first time, it is clearly demonstrated — using both a syngeneic mouse mammary tumor model (employing 4T1-*Luc2* in immunocompetent BALB/c mice), as well as a complementary xenograft tumor model (employing human 2Lmp/*Luc* breast cancer cells) — that such approaches are directly relevant to breast cancer.

Second, and particularly important from a clinical perspective, we establish that ex vivo expanded $\gamma\delta$ -T cells are not only effective against disease, but also do not cause untoward side effects upon adoptive transfer into tumor-bearing hosts. This is a key point to be made as clinical trials are developed to assess how $\gamma\delta$ -T cells might best be administered therapeutically. Thus, we show that syngeneic BALB/c-derived $\gamma\delta$ -T cells — which are capable of killing 4T1 cells

in vitro and in vivo (Figures 1 and 4) — are nevertheless unreactive against normal BALB/c fibroblasts in vitro (Figure 1). Moreover, these ex vivo expanded $\gamma\delta$ -T cells are well-tolerated by mice receiving treatments, even when delivered in multi-dose schedules. Similarly, from our studies shown in Figure 5, we infer that adoptively-transferred human $\gamma\delta$ -T cells do not react with non-malignant (albeit, xenogeneic) tissues in vivo as these mice also tolerated multi-dose treatments well. This particular conclusion is indirectly supported by our previous work showing that in vitro, non-malignant human cell lines are not killed by ex vivo expanded human $\gamma\delta$ -T cells which, nevertheless, readily recognized and killed human breast cancer cell lines [17].

The third objective of this current work is to highlight one of the potential advantages of adopting the approach whereby $\gamma\delta$ -T cells are first expanded ex vivo, then reinfused. In contrast to the alternative approach whereby endogenous $\gamma\delta$ -T cells are activated in vitro within patients, in using the approach taken here, it becomes possible for investigators to experimentally track $\gamma\delta$ -T cells after administration — an important correlative tool for use in the design, interpretation and refinement of future clinical trials. Thus, in a manner analogous to our animal studies using ^{111}In -labeled $\gamma\delta$ -T cells (Figures 2 and 3), it will be possible to track therapeutically-administered human $\gamma\delta$ -T cells to sites of disease employing the appropriate clinically-approved imaging techniques [31]. Moreover, in early human clinical trials which we are about to embark upon at our institution (UAB Breast Cancer SPORE Project 4, “Gamma-delta T cell Immunotherapy of Breast Cancer; Project co-leaders, R. Lopez, K. Zinn), the optimal $\gamma\delta$ -T cell dose and schedule remain to be determined. Accordingly, in a manner similar to the studies presented in Figure 3, we will be able to perform critical clinical biodistribution studies, an important first step in the optimization of $\gamma\delta$ -T cell-based immune therapies.

The observation that ^{111}In -labeled $\gamma\delta$ -T cells readily localize to mammary tumors is not surprising (Figure 2), especially given our previous report that adoptively-transferred $\gamma\delta$ -T cells readily localize into tumors in a mouse model of prostate cancer [26]. Moreover, the finding that this localization to tumor appears to be only partially reduced by an anti- $\gamma\delta$ -TCR antibody (mAb GL3) is consistent with our previous findings that in vitro, antibodies to the $\gamma\delta$ -TCR only partially inhibit binding of $\gamma\delta$ -T cells to sensitive tumor cell targets [27]. In any event, the altered homing displayed by antibody-treated $\gamma\delta$ -T cells could reflect disrupted trafficking, or alternatively, antibody-treated $\gamma\delta$ -T cells could display altered survival, be eliminated by virtue of being coated with antibody, or exhibit impaired proliferation within the local tumor site. This issue is currently under investigation.

Intriguingly, in the biodistribution studies (Figure 3), adoptively-transferred $\gamma\delta$ -T cells were found to be more abundant in the spleens of healthy mice when compared to the spleens of tumor-bearing mice. Although we have no clear explanation for this finding, we surmise that $\gamma\delta$ -T cells in tumor-bearing mice are trafficking differently — possibly as a result of altered homing receptors expressed on lymphoid tissues, including the spleen, within tumor-bearing mice. Conversely, intrinsic changes within the $\gamma\delta$ -T cells may account for their observed altered biodistribution in tumor-bearing mice. We are currently undertaking studies to address this issue, as findings from such studies could have practical and clinical implications in the conduct of $\gamma\delta$ -T cell-based therapeutic trials.

Given the biological, technological and pharmaceutical advances of the last several years, human clinical trials intended to exploit the innate antitumor properties of $\gamma\delta$ -T cells are now a reality [10,11,23,42]. Indeed as noted above, at our institution, we are preparing to embark upon our first generation of clinical trials — including a phase I trial in which patients with advanced breast cancer are to be treated with peripheral blood-derived autologous $\gamma\delta$ -T cells first ex vivo expanded, then subsequently reinfused. Accordingly, in the performance of such

early phase trials, a more thorough understanding of the in vivo activity and fate of adoptively-transferred $\gamma\delta$ -T cells will be key to refining later generations of trials. In this context, the timeliness and relevance of our current findings are underscored.

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Fig. 1.

Ex vivo expanded BALB/c-derived $\gamma\delta$ -T cells kill syngeneic 4T1 mammary adenocarcinoma cells but not syngeneic normal fibroblasts. $\gamma\delta$ -T cells were obtained and expanded from the spleens of BALB/c TCR $\alpha\beta^{-/-}$ mice. FACS-plot inset shows representative purity of ex vivo expanded $\gamma\delta$ -T cells employed in the in vitro cytotoxicity assays (and adoptive transfer studies). BALB/c-derived effector $\gamma\delta$ -T cells were co-cultured with syngeneic target cell lines 4T1 (BALB/c mammary adenocarcinoma) and BALB/3T3 (normal BALB/c fibroblast cell line) at a 10:1 effector:target ratio for 4 hours in a standard cytolytic assay. Cytotoxicity was determined and is expressed as percent specific lysis $\pm$ standard deviation. These results are from experiments performed three separate times

Fig. 2.

Adoptively-transferred $\gamma\delta$ -T cells localize to 4T1 tumors. SPECT/CT fused images (axial view) showing distribution of ^{111}In -labeled $\gamma\delta$ -T cells in 4T1 mammary fat pad tumors at 48 hours post injection. To demonstrate that the $\gamma\delta$ -T cell receptor ($\gamma\delta$ -TCR) itself is involved in the localization of adoptively-transferred cells to tumors, imaging was performed using ^{111}In -labeled $\gamma\delta$ -T cells which were either (a) untreated or (b) pre-treated with an anti- $\gamma\delta$ TCR monoclonal antibody (clone GL3) before injection into mice. Dotted circles in each image delineates tumor region. These results are representative of experiments performed on at least three separate mice in each group

Fig. 3.

Adoptively-transferred $\gamma\delta$ -T cells distribute differently within tumor-bearing mice as compared to healthy mice. ^{111}In -labeled $\gamma\delta$ -T cells were injected into healthy mice or tumor-bearing mice. Biodistribution of administered $\gamma\delta$ -T cells (expressed as a percent of injected radioactive dose per gram of target tissue) was determined by resecting tissues or organs isolated from healthy BALB/c mice (■; $n = 10$) or from BALB/c mice harboring 4T1 cells (□; $n = 10$). Tissues: HT, heart; LV, liver; ST, stomach; LI, large intestine; SI, small intestine; CE, cecum; SP, spleen; LU, lung; LK, left kidney; RK, right kidney; MU, muscle; BL, blood; RO, reproductive organs; BR, brain; FE, femur. These results are representative of experiments performed two separate times. Statistically significant differences between tissues taken from healthy mice or from tumor-bearing mice are indicated by asterisks ($p = 0.003$ for spleen)

Fig. 4.

Bioluminescence studies: In vivo sensitivity of murine mammary adenocarcinoma cancer cell line 4T1-*Luc2* to killing by adoptively-transferred syngeneic BALB/c $\gamma\delta$ -T cells. 4T1-*Luc2* cells were injected into the mammary fat pads of healthy female BALB/c mice. After 2 days, all animals were imaged and matched pairwise based on equivalence of tumor burden. Each animal from a given pair was then randomly assigned to a group receiving either treatment or no treatment. Treated animals (n = 9) received 5×10^6 BALB/c-derived $\gamma\delta$ -T cells intravenously on days 2, 5 and 9. Untreated animals (n = 9) received sham-injections with saline. Whole animal images of tumor-bearing mice (19 days post-tumor injection) were obtained using an IVIS Imaging System Series 100 bioluminescence detector (Xenogen) 10 minutes after intraperitoneal injection of 2.5 mg luciferin (substrate for luciferase). Images of four representative untreated mice (panel **a**, top) and four representative treated mice (panel **b**, top) are shown. Light emission from tumor cells was measured using Living Image 3.1 software and is represented as a pseudo-color scaling of the bioluminescence data. Bioluminescence data are shown graphically where tumor burden is expressed in counts per second. Mean ($\pm$ SD) and median tumor burdens of untreated and treated mice are shown. In these studies, diminished luciferase activity in tumor-bearing animals treated with syngeneic BALB/c-derived $\gamma\delta$ -T cells is taken as evidence of in vivo $\gamma\delta$ -T cell anti-tumor efficacy

Fig. 5.

Bioluminescence studies: In vivo sensitivity of human breast cancer cell line 2LMP/*Luc* to killing by human $\gamma\delta$ -T cells. Human breast cancer cell line 2LMP/*Luc* was derived by transducing 2LMP cells to express firefly luciferase using the recombinant adeno-associated virus-2 transduction methods [7,32-35]. 2LMP/*Luc* cells (1×10^6 cells) were introduced subcutaneously into 10 nude mice on day 0. After 6 days, all animals were imaged and matched pairwise based on equivalence of tumor burden. Each animal from a given pair was then randomly assigned to a group receiving either treatment or no treatment. Treated animals received 20×10^6 human $\gamma\delta$ -T cells intravenously on days 6, 9, 13, 16, 20 and 23. Untreated animals received sham-injections. Serial images from a representative pair of mice are shown here (panel **a**, untreated mouse above; treated mouse below). A graphic representation of bioluminescence data expressed in counts per second is shown to the right of the images. Normalized data from all animals are shown in panel **b**. For each animal, all measurements of tumor size (in counts per second) were normalized to tumor size determined for that animal on day 6 after tumor implantation (panel **b**). Thus on day 6, each animal has a “measured tumor size to initial tumor size” ratio of 1. Data from subsequent days are expressed as a ratio of “measured tumor size to initial tumor size” (mean $\pm$ SD). Open bars, untreated animals ($n=5$). Solid bars, treated animals ($n=5$). In these studies, diminished luciferase activity in tumor-bearing animals treated with human $\gamma\delta$ -T cells is taken as evidence of in vivo human $\gamma\delta$ -T cell antitumor activity