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Immunotherapy Of Cancer Employing γδ-T Cells: A Study Examining Their Utility And Feasibility

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IMMUNOTHERAPY OF CANCER EMPLOYING γδ-T CELLS: A STUDY EXAMINING THEIR UTILITY AND FEASIBILITY

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IMMUNOTHERAPY OF CANCER EMPLOYING γδ-T CELLS: A STUDY EXAMINING THEIR UTILITY AND FEASIBILITY

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PATHOLOGY

ABSTRACT

Unlike antigen-specific αβ-T cells, γδ-T cells can recognize and lyse cancerous cells rapidly upon encounter in a manner that does not require the recognition of tumor-specific antigens. Given the well-documented capacity of γδ-T cells to innately kill malignant cells, efforts are now underway to exploit the antitumor properties of γδ-T cells for clinical purposes.

Here, we present for the first time preclinical in vivo mouse models of γδ-T cell-based immunotherapy directed against breast cancer. These studies were explicitly designed to approximate clinical situations in which adoptively-transferred γδ-T cells would be employed therapeutically against breast cancer. Using radioisotope-labeled γδ-T cells, we show that adoptively-transferred syngeneic γδ-T cells localize to breast tumors in a mouse model of breast cancer. Moreover, in both syngeneic and xenogeneic models of breast cancer, we demonstrate that adoptively-transferred γδ-T cells are both effective against breast cancer and are well-tolerated by treated animals. These findings provide a strong preclinical rationale for using ex vivo expanded adoptively-transferred γδ-T cells for the treatment of breast cancer.

Additionally, we investigated a critical issue surrounding γδ-T cell cancer immunotherapy, which relates to the findings that only in some cancer patients is it possible to activate and/or expand γδ-T cells either *in vivo* or *ex vivo* regardless of the methodology employed. This is in stark contrast to what is observed in healthy

individuals, as γδ-T cells reliably respond to proliferative stimuli. To investigate this issue, we attempted the expansion of γδ-T cells from tumor-bearing mice, instead of healthy mice (as performed in the studies mentioned above) for the adoptive-transfer into tumor-bearing hosts. We found that γδ-T cells in tumor-bearing mice were numerically rare and expanded poorly ex vivo and that tumor cells were responsible for this numerical and functional exhaustion of γδ-T cells. Using both *in vitro* and *in vivo* models of different cancers we demonstrate that γδ-T cells undergo apoptosis after encounter with tumor cells and we identified several putative genes involved in this exhaustion of γδ-T cells. These findings will facilitate the development of the next generation of clinical trials exploiting the tumor-reactive properties of γδ-T cells.

Keywords: γδ-T cell, cellular immunotherapy, innate immunity

DEDICATION

This work is dedicated to my family. To my wife Lauren, thank you for your endless love and support during this trying period of our lives. To my daughter Sipsey, thank you for opening up my eyes to the world. To my parents, Marlene and Artie, thank you for the encouragement throughout the years. You have always been supportive. Finally, in memory of my father-in-law Mark, I miss you dearly and promise to take care of your girls. I love you all.

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I would also like to thank my lab colleagues who helped me along the way: Zhiyong Liu, Ben Guo, Hilary Haines, and Hyung Kim. Each of you taught me much and I always enjoyed your company.

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TUMOR CELL DRIVEN EXHAUSTION OF THE γδ-T CELL COMPARTMENT: IMPLICATIONS FOR THE IMMUNOTHERAPY OF MALIGNANCIES

INTRODUCTION

γδ-T cells link adaptive and innate immunity

Historically, the immune system has been divided into two arms. Innate immunity, the more ancestral arm, is composed of effector cells that can rapidly recognize and respond to a variety of foreign agents typically based upon their molecular patterns. The other arm, adaptive immunity, consists of T and B lymphocytes bearing highly specific antigen receptors that generate an exceedingly coordinated and specific response, although at a much slower pace than the innate system. γδ-T cells are unique in that they are thought to be the cellular link or bridge between both responses. More specifically, γδ-T cells are part of the adaptive immune system because they can acquire a memory phenotype and possess junctionally diverse T cell receptors (TCR) that require gene arrangement [45]. However, γδ−T cells are also a component of the innate immune response, because they are non-major histocompatibility complex (MHC)-restricted, and they express a restricted TCR repertoire which they use as a pattern recognition receptor [45].

γδ−T cell phylogeny, nomenclature, and tissue distribution

The γδ−TCR is highly conserved, with the genes encoding the receptor found in primates, ruminants, rodents, birds, cartilaginous fish, and teleost fish [64, 69]. Intriguingly, γδ-T cell abundance varies greatly between species; in mice and humans γδ-

T cells represent 1-5% of circulating T cells, yet γδ-T cells account for ≥20% in birds and ≤70% in young ruminants [14, 43, 58, 69].

In humans, there are two predominant subsets of γδ-T cells, the Vγ9Vδ2 subset, which are most commonly found in the peripheral blood; and the lesser-studied Vδ1 subset (pairs with different Vγ chains), which are predominant in the intestinal intraepithelial compartments. In mice, there are six principal subsets of γδ-T cells and unlike humans, mice have a substantial representation of γδ-T cells residing within the epidermis known as dendritic epidermal T cells (DETCs).

Curiously, throughout phylogeny, γδ-T cells are rarely found in conventional T cell areas such as the lymph node parenchyma, white pulp of the spleen, Peyer's patches, and thymus [15, 41, 43]. Instead γδ-T cells are localized to tissues and are found disproportionately abundant in the intestine [15, 41, 43]. In addition, murine γδ-T cells develop in the thymus in waves and sequentially populate different tissues [3, 41, 69]. Therefore, at distinct embryonic timepoints, thymocytes bear a unique γδ-TCR and subsequently populate specific tissues [3, 41, 69].

It is important to note here that there are two different nomenclature designations [31, 42] for murine γδ-T cells and that we use the nomenclature designated by Heilig and Tonegawa (1986).

The γδ-T cell: The quintessential jack-of-all-trades

γδ-T cells play crucial roles in immunity including primary immune responses such as anti-microbial activity, along with well-accepted roles in tumor immunosurveillance, and immunoregulation. With respect to their anti-microbial

activity, the primary literature is rich with descriptions of γδ-T cell responses to a myriad of bacterial, viral, and protozoan pathogens and pathogen-infected cells. In humans, a large-scale activation and expansion of γδ-T cells bearing the Vγ9Vδ2 receptor is observed after infection with a broad range of microbial pathogens. Moreover, γδ-T cells exhibit robust responses *in vitro* to pathogen derivatives [61]. Collectively, it is now well-established that γδ-T cells are crucial for the regulation of immune responses in a number of infectious disease states including tuberculosis, malaria, and viral diseases such as human immunodeficiency virus (HIV), herpes simplex virus (HSV), and cytomegalovirus (CMV) [16, 27, 44, 53, 70, 72].

The γδ-T cell response to pathogens, while important, is not the focus of the work in this dissertation. Instead, the dissertation studies reported here focus on another property of γδ-T cells, their ability to kill tumor cells. However, an understanding of the anti-pathogen responses of γδ-T cells is crucial in transitioning to the discussion of their inherent anti-tumor properties because the means by which γδ-T cells recognize both pathogens and tumor cells appears to be remarkably similar and will be expounded upon below.

Distinguishing friend from foe

While conventional αβ-T cells utilize their T cell receptor to recognize specific antigenic peptides within the context of an MHC molecule, γδ-T cells can recognize antigen in an MHC class-I or class-II independent manner. Antigen recognition occurs through the γδ-TCR or in a TCR-independent manner through a natural killer like receptor termed NKG2D.

Broadly, γδ-T cells have been shown to directly recognize and respond to determinants of cellular stress. A comprehensive identification of γδ-T cell antigens remains enigmatic, however, many have been identified and rapid progress is underway. Human Vγ9Vδ2 T cells as well as simian γδ-T cells (*but not murine γδ-T cells*) are activated by small non-peptidic phosphorylated compounds referred to as phosphoantigens [8]. In all instances, these molecules are metabolites of the isoprenoid biosynthesis pathway. Isoprenoids include such diverse molecules as sterols, dolichols, plastoquinones, uniquinones, carotenoids, and the prenyl side chains of chlorophylls [27]. In fact, the most potent Vγ9Vδ2 T cell activator known is (E)-4hydroxy-3-methyl-but-2-enyl-pyrophosphate (HMB-PP), which is an intermediate of the 2-*C*-methyl-D-erythritol-4-phosphate (MEP) pathway of isoprenoid biosynthesis utilized by many pathogenic bacteria [27]. In contrast, the activation of Vγ9Vδ2 cells by metabolites produced through the mevalonate pathway — the isoprenoid synthesis pathway used by mammalian cells — such as isopentenyl pyrophosphate (IPP), while substantial, is about 10,000-fold lower than HMB-PP [8].

Interestingly, because human γδ-T cells are activated by IPP, this trait has led to the therapeutic exploitation of γδ-T cells through the use of drugs that induce an intracellular accumulation of IPP within tumor cells; namely the bisphosphonate class of drugs. In particular, aminobisphosphonates such as pamidronate and zoledronate — drugs that are better known to be used in the treatment of osteoporosis and tumor-associated bone diseases — have been shown to inhibit farnesyl pyrophosphate synthase (FPPS), which results in the accumulation of IPP within tumor cells and consequently the activation of human Vγ9Vδ2 cells [49, 79]

Taken as a whole, these findings establish an evolutionary connection of sorts between the recognition of microbes and tumor cells because Vγ9Vδ2 T cells ultimately recognize exogenous or endogenous prenyl pyrophosphates in isoprenoid pathways [62]. In other words, γδ-T cells evolved to respond to a class of antigens or chemical features so broad they span the gamut from prokaryotes to malignantly-transformed cells.

Other well-defined ligands of the γδ-TCR consistently fall into a group of self-molecules that indicate cellular stress [10]. Such ligands include heat shock proteins (HSP) which are protein chaperones that are typically localized within the mitochondrion, but during times of cellular stress are frequently aberrantly expressed and subsequently act as potent activators of γδ-T cells [9, 29, 66, 67]. Another mitochondrial protein, F1-ATPase, is expressed on the surface of some tumor cells whereby it promotes Vγ9Vδ2 recognition and activation [76]. Intriguingly, similar to the phosphoantigens, γδ-T cell recognition of HSPs could be related to the putative microbial origin of mitochondria, which begs the question as to whether HSPs display resemblance to microbial antigens.

As mentioned above, γδ-T cells can also recognize antigen with natural killer-like receptors, notably the receptor NKG2D. Indeed, many of the published reports demonstrating γδ-T cell-mediated in vitro cytotoxicity to tumor cell targets is attributed to recognition of tumor targets through the NKG2D receptor. Ligands for NKG2D include UL16-binding proteins and MICA and MICB, which collectively are MHC-class I related proteins that are known to be generic indicators of cellular stress [59, 86].

With regards to effector functions, or in this case eliminating foe, it remains unclear whether γδ-T cells en masse have a predominant effector function; however, γδ-T cells share many effector mechanisms with αβ-T cells including fas/fasL interactions,

cytolytic granule production (e.g., granulysins and perforins) and the production of proinflammatory cytokines like IFN-γ [41].

However, γδ-T cells are not exclusively pro-inflammatory and cytotoxic in disposition. In contrast, a role for γδ-T cells in immunoregulation has been demonstrated in numerous studies. If truth be told, immunoregulation seems to be the least understood capacity of γδ-T cell biology because γδ-T cells in this context are profoundly pleiotropic. So much so, that the questions are simply outpacing the answers.

For instance, in cutaneous wound healing studies, γδ-T cells (specifically DETCs) were shown to produce keratinocyte growth factor (KGF) and other chemokines suggesting a role for γδ-T cells in tissue repair [46]. Moreover, mice deficient in γδ-T cells exhibited profound defects in keratinocyte proliferation and tissue reepithelialization [46]. Recently, γδ-T cell deficient mice were reported to frequently develop a spontaneous inflammation in the cornea of the eye suggesting a role for γδ-T cells in maintaining the immune balance in the eye [68].

In contrast, γδ-T cells may also play a role in exacerbating certain diseases. In experimental autoimmune encephalitis (EAE) — a murine model of multiple sclerosis (MS) — γδ-T cells were found to rapidly migrate to the injection site of the EAE-inducing insult where they underwent a massive expansion and were thought to be critical contributor of EAE development [84, 85]. Likewise, during collagen-induced arthritis (CIA) — a murine model of rheumatoid arthritis — γδ-T cells exacerbate CIA, notably through the production of IL-17 [71].

An increasing layer of complexity surrounding γδ-T cells is afforded by virtue of their supposed ability to present antigen [12]. Indeed, human Vγ9Vδ2 T cells can take up

and process soluble proteins and induce proliferation, target cell killing, and cytokine production responses in antigen-experienced and naïve $CD8^+$ αβ-T cells [12].

Cell-based immunotherapy of cancer: current understanding and limitations

The view that the immune system can recognize and kill malignantly-transformed cells is not new. In fact, over fifty years ago the hypothesis of cancer immunosurveillance was proposed at approximately the same time by Burnet and Thomas [17, 26]. In 1957, Burnet stated:

> "A slightly more hopeful approach (*over chemotherapy*) is so dependent on the body's own resources that it has never been seriously propounded, is the immunological one. It is generally regarded as axiomatic that, since a cancer cell is of the body's own pattern, no effective immunological action against it is possible. In view of recent work, it is, however, conceivable that in many instances there is sufficient antigenic difference to be effective [17]."

In addition, Burnet discussed:

> "It is by no means inconceivable that small accumulations of tumour cells may develop and because of their possession of new antigenic potentialities provoke an effective immunological reaction with regression of the tumour and no clinical hint of its existence [17]."

Soon after, the view that the immune system could recognize and eliminate transformed cells was repeatedly challenged, nearly nullified, and has since been resurrected. To be sure, since Burnet's time, the idea that cellular immune responses to treat malignancies has been the focus of numerous studies. To date, the majority of studies in this regard have focused primarily upon exploiting adaptive cellular immune responses directed against tumor-specific or tumor-associated antigens. This includes a number of important studies designed to generate tumor-specific cytotoxic CD8+ αβ-T

lymphocytes (CTL) utilizing specific peptide antigens, as well as other studies designed to develop tumor-specific immune responses employing dendritic cell-based vaccination strategies [22, 36, 65, 75]. However, these and similar approaches which rely upon adaptive immunity suffer from several potential shortcomings.

First, these strategies presume that an antigen selected as a target for cell-based immunotherapy is indeed tumor-specific—that is, the antigen is uniquely expressed by tumor cells, and not expressed by normal tissues. Second, various antigens which might serve as therapeutic targets may not be ideal on account that they may be expressed only by a proportion of malignant cells, which would ultimately result in the selective outgrowth of tumor cells that do not express the unique antigen. Furthermore, while it may be feasible to identify and stimulate tumor-antigen-specific responses, particularly for malignancies with high immunogenicity such as melanoma, the tumor cell population simply does not remain static in the face of the anti-tumor force exerted by effectors such as αβ-T cells [33]. Thus, in due course immunologic pressures such as these may serve to edit or sculpt the immunogenicity of tumor cells and eventually lead to their complete escape from immunological attack [26].

Theoretical rationale for employing γδ-T cell-based immunotherapies against cancer

With this in mind, particularly in the context of developing novel cell-based approaches for the treatment of malignancies, it becomes especially important to consider and explore tumor antigen-independent (innate) cellular immune responses mediated by such cells as γδ-T cells or other innate effectors like natural killer (NK) cells.

As mentioned above, in contrast to αβ-T cells, γδ-T cells can directly recognize and respond to tumor cells in an MHC-independent manner through less specific mechanisms that require no previous antigen exposure or priming (such as is required in adaptive immunity). Instead, γδ-T cells recognize generic antigens, which can be expressed by stressed cells including cells which have undergone malignant transformation. To be sure, cancerous cells are now known to 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 γδ-T cells [18, 32, 33, 37, 38, 49]. Because of their ability to directly recognize and respond to tumor cells, it stands to reason that γδ-T cells may be complementary or even advantageous in some ways to the adaptive arms of the immune system for the cell-based immunotherapy of cancer.

Antitumor activity of γδ-T cells and tumor immunosurveillance: findings from animal studies

Several studies in mice have demonstrated the capacity of γδ-T cells to regulate malignancy. Many of these studies have utilized the TCRδ$^{-/-}$ mouse, which lacks all γδ-T cells. Tumors form more readily in γδ-T cell deficient mice compared to wild-type mice after injection of squamous-cell carcinoma or melanoma tumor cell lines, or after subjecting mice to cutaneous carcinogenesis regimens [30, 34, 78]. Recently, our laboratory has confirmed and extended these important findings by demonstrating that γδ-T cells provide some degree of in vivo tumor immunosurveillance against murine prostate cancer [50]. Using the TRAMP transgenic mouse model of prostate cancer, we have shown that the absence of γδ-T cells is permissive for the development of tumors as

demonstrated in TRAMP mice, which develop prostate cancer in a predictable manner [54]. By back-crossing TRAMP mice with mice lacking γδ-T cells (TCRδ$^{-/-}$), we show that TRAMP × TCRδ$^{-/-}$ mice develop more rapid and aggressive prostate cancers compared to age-matched TRAMP mice. In addition, we demonstrated that adoptively-transferred syngeneic γδ-T cells are therapeutically effective against established tumors from TRAMP cell lines (specifically the TRAMP-C2 cell line). Furthermore, in murine xenograft models, human γδ-T cells expanded ex vivo have been shown to reduce tumor burden and improve survival when injected into SCID mice harboring human melanoma or pancreatic cancer cells [50].

Antitumor activity of γδ-T cells and tumor immunosurveillance: findings from preclinical/clinical studies

Several factors suggest that γδ-T cell-based immunotherapies could be applicable to a wide variety of human cancers. First, there is an exhaustive number of reports demonstrating that human γδ-T cells can indeed recognize and kill a wide variety of malignant human cell lines ranging from those of epithelial origin (e.g., breast, prostate, colorectal, pancreatic, lung, glioblastoma and other cell lines), to include those of hematolymphoid origin as well (lymphoma and myeloma cell lines) [2, 11, 13, 19, 40, 55, 63, 74, 80, 86]. Second, reports have shown that γδ-T cells isolated from tumors removed from patients (i.e., tumor-infiltrating lymphocytes) retain *in vitro* lytic activity against human cancer cells, yet almost uniformly fail to kill non-malignant human cell lines which is a favorable, if not necessary trait, particularly in the context of studies in which γδ-T cells are to be administered therapeutically [19, 80].

Given the well-accepted capability of γδ-T cells to innately kill a variety of cancerous cells, efforts are now actively underway to develop and refine the means to exploit the antitumor properties of γδ-T cells for clinical purposes [7, 20, 21, 51, 83]. Currently, two approaches are being developed in an attempt to capitalize on the well-accepted antitumor properties of γδ-T cells. One approach attempts the activation or expansion of the endogenous repertoire of γδ-T cells within patients through the clinical administration of pharmacologic agents capable of stimulating γδ-T cells, such as aminobisphosphonate drugs, typically in concert with cytokines such as IL-2 [20, 21, 52, 77, 83]. In another approach, γδ-T cells are isolated from tumor-bearing patients, expanded ex vivo, and adoptively re-infused into the patient [51, 54].

While both approaches are clearly rational and may lead to significant advances in cancer treatment, an increasingly apparent problem with these approaches is emerging. This problem relates to γδ-T cells themselves found within patients. It was first reported that when compared to healthy donors, endogenous γδ-T cells are substantially fewer in numbers in the peripheral blood of patients newly diagnosed with certain cancers [5]. In our laboratory, we have confirmed and extended these clinical findings by documenting numerical deficits of γδ-T cells in the peripheral blood of glioblastoma, prostate, breast, and lung cancer patients (manuscripts in preparation) (Figure 1).

Additionally, and perhaps more importantly, it appears that only in a proportion of patients is it possible to efficiently activate or expand patient-derived γδ-T cells either in vivo or ex vivo, which is a stark contrast to what is observed in normal, healthy individuals where robust activation or expansion is reliably achievable. For example, reports show that the ex vivo expansion of γδ-T cells derived from melanoma and

nasopharyngeal carcinoma (NPC) patients is significantly impaired compared to γδ-T cells derived from healthy subjects [5, 87]. In another study, patients with hematologic cancers exhibited impairments in γδ-T cell ex vivo proliferation so severe, it necessitated the "pre-screening" of study subjects for adequate in vitro γδ-T cell proliferation before their enrollment in clinical trials [83].

Focus of this dissertation

The dissertation studies undertaken here were performed with two distinct yet logically related objectives in mind. Previously, our laboratory demonstrated that ex vivo expanded human γδ-T cells were capable of effectively killing human breast cancer cell lines in vitro [40]. Moreover, clinical studies reported that γδ-T cells accumulate within breast tumors and adjacent draining lymph nodes [1, 6].

While the previous findings by ourselves and others have been important in establishing the theoretical potential of γδ-T cell-based immunotherapy for the treatment of breast cancer, such studies have been either observational or limited to in vitro studies. Thus, at the start of my dissertation studies (the first chapter of this dissertation) our first objective was to examine the efficacy and safety of employing adoptively-transferred tumor-reactive γδ-T cells for the immunotherapy of breast cancer. We hypothesized that ex vivo expanded adoptively-transferred γδ-T cells could control the growth of mammary tumor cells in two different murine models of breast cancer. Ultimately, this arm of dissertation studies was explicitly designed to demonstrate the utility of γδ-T cells for the immunotherapy of breast cancer in an effort to provide a strong biological rationale to

facilitate a move into phase-I clinical trials exploiting γδ-T cells for their anti-tumor properties in the treatment of advanced or recurrent breast cancer.

Our next objective was to examine a potential limitation to the utilization of γδ-T cells for immunotherapeutic purposes; this limitation (as described in the above Introduction) arises from clinical studies reporting an intrinsic exhaustion of γδ-T cells within or derived from patients. Thus, in the latter section of this dissertation we attempted to corroborate these clinical observations. Specifically, we hypothesized that tumor cells may be responsible for, or contribute to, this exhaustion of γδ-T cells. To directly assess this issue, we utilized animal models of different malignancies to explore the mechanism(s) explaining these defects in the γδ-T cell compartment.

Collectively, these dissertation studies are important for two reasons. Foremost, these studies are important in demonstrating the utility of γδ-T cell-based immunotherapy for the treatment of breast cancer. Second, these studies are rooted in basic biology as they provide new biological insights about γδ-T cells. Together these findings will help to further our understanding of γδ-T cells and in the end we hope these findings will positively impact human lives.

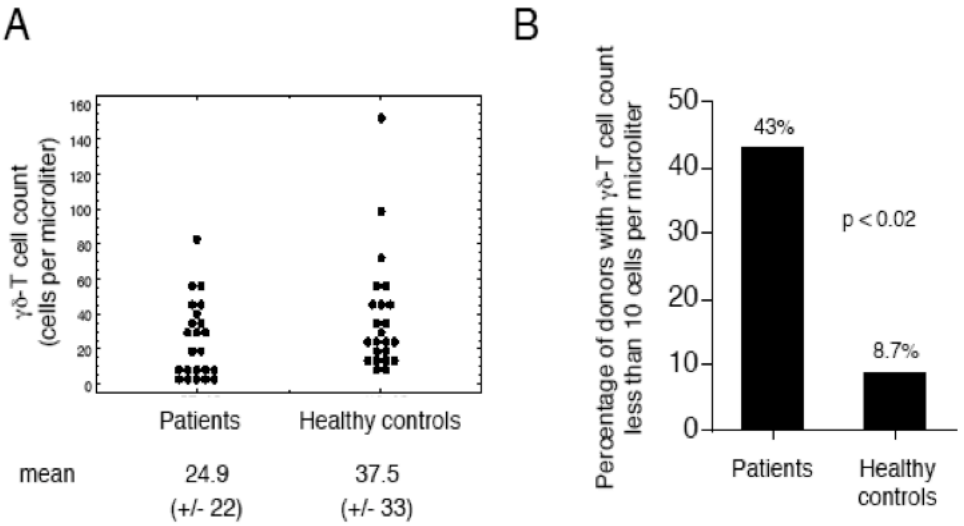

Figure 1. Prostate cancer patients exhibit a numerical deficit of γδ-T cells in peripheral blood. From each blood donor, a complete blood count was obtained using a Coulter LH 750 analyzer. Aliquots of whole blood were stained for flow cytometric analysis using anti-γδ-TCR-FITC and CD3-APC and the appropriate isotype control reagents. Absolute counts of γδ-T cells (expressed as γδ-T cell count in cells per microliter) were calculated by multiplying the whole blood lymphocyte count (obtained from Coulter counter) by the percentage of γδ-T cells found within the lymphocyte gate as determined by flow cytometric analysis. The mean γδ-T cell count (cells/microliter +/- SD) are shown for prostate cancer patients and healthy donors.

ADOPTIVELY-TRANSFERRED EX VIVO EXPANDED γδ-T CELLS MEDIATE IN VIVO ANTITUMOR ACTIVITY IN PRECLINICAL MOUSE MODELS OF BREAST CANCER

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ABSTRACT

In contrast to antigen-specific αβ-T cells (adaptive immune system), γδ-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 γδ-T cells to innately kill a variety of malignant cells, efforts are now actively underway to exploit the antitumor properties of γδ-T cells for clinical purposes. Here, we present for the first time preclinical in vivo mouse models of γδ-T cell-based immunotherapy directed against breast cancer. These studies were explicitly designed to approximate clinical situations in which adoptively-transferred γδ-T cells would be employed therapeutically against breast cancer. Using radioisotope-labeled γδ-T cells, we first show that adoptively-transferred γδ-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 γδ-T cell receptor (TCR) we determined that localization of adoptively-transferred γδ-T cells to tumor is a TCR-dependent process. Additionally, biodistribution studies revealed that adoptively-transferred γδ-T cells traffic differently in tumor-bearing mice compared to healthy mice with fewer γδ-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 γδ-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 γδ-T cells as a form of cell-based immunotherapy for the treatment of breast cancer. Additionally, these studies es-

tablish that clinically-applicable methods for radiolabeling γδ-T cells allows for the tracking of adoptively-transferred γδ-T cells in tumor-bearing hosts.

INTRODUCTION

Unlike αβ-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), γδ-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, γδ-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, γδ-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 γδ-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 γδ-T cells for clinical purposes [4, 10, 11, 23, 42]. Over a decade ago, studies documented the presence of γδ-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 γδ-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 γδ-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 γδ-T cell-based immunotherapy directed against breast cancer. These studies were explicitly designed to approximate clinical situations in which adoptively-transferred γδ-T cells would be employed therapeutically against breast cancer. Using radioisotope-labeled γδ-T cells, we show that adoptively-transferred γδ-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 γδ-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 γδ-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 αβ-deficient (TCRαβ$^{-/-}$) 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 γδ-T cells

γδ-T cells used in cytotoxicity assays and immunotherapy studies were obtained from spleen cells derived from BALB/c mice lacking αβ-T cells (TCRαβ$^{-/-}$) as previously described [28]. Briefly, whole spleens were resected from TCRαβ$^{-/-}$ 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 γδ-T cells for xenograft studies were prepared as previously described [29].

Flow cytometry

To assess purity of γδ-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-γδ-FITC (clone GL3), anti-TCR-γδ-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 γδ-T cells against 4T1 and BALB/3T3 cell lines was measured using the standard ^{51}Cr 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 γδ-T cell killing in vitro (data not shown).

Biodistribution studies using ^{111}In-labeled γδ-T cells

Eight-week old female BALB/c mice were sham-injected with saline, or received 4X10^4 4T1 mammary adenocarcinoma cells in the mammary fat pad. After 20 days, mice were injected intravenously with 5X10^6 BALB/c-derived ^{111}In-labeled γδ-T cells, labeled with the standard nuclear pharmacy ^{111}In-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 γδ-T cell localization to tumor, γδ-T cells were first pretreated (prior to injection) with 10 μg/ml anti-γδ-TCR monoclonal antibody clone GL3 for 15 minutes on

ice. The GL3 antibody has been shown to downregulate the γδ-TCR, which acts to functionally impair γδ-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 γδ-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 γδ-T cells), tumors were first established in otherwise healthy mice. In these studies, $4X10^4$ 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 γδ-T cells ($5x10^6$ 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 γδ-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 γδ-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 γδ-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 γδ-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 γδ-T cells, indicating that γδ-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 γδ-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) γδ-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) γδ-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 γδ-T cells might be used in the treatment of breast cancer.

Adoptively-transferred γδ-T cells localize to tumors.

We next designed a series of studies to determine the extent to which adoptively-transferred γδ-T cells could localize within primary mammary tumors. Previously, using

a syngeneic mouse model of prostate cancer, we showed that γδ-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 γδ-T cells would be feasible. Accordingly, in this present study, we employed radiolabeled (^{111}In) γδ-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 γδ-T cells do indeed localize to 4T1 mammary fat pad tumors. Importantly, when γδ-T cells were first pretreated (prior to injection) with an anti-γδ-TCR monoclonal antibody which is known to downregulate the γδ-TCR [20, 24], a marked decrease in localization of γδ-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 γδ-T cells, which were either pretreated (blocked) with the anti-γδ-TCR antibody GL3 antibody, or left untreated (unblocked). Tumors were then resected from these mice. As a quantitative measure of γδ-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 γδ-T cells, a 34.6% reduction in γδ-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 γδ-T cells, either trafficking or the functional accumulation within tumor is to some degree dependent on the expression of a functional γδ-TCR.

γδ-T cell trafficking patterns differ between healthy and tumor bearing mice.

The demonstration that adoptively-transferred γδ-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 γδ-T cells. In these studies, both healthy BALB/c mice and 4T1-bearing BALB/c mice were injected with 5 x 10^6 ^{111}In-labeled-γδ T cells and compared. After 48 h, mice were sacrificed and tissues were removed to assess the distribution of adoptively-transferred γδ-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 γδ-T cells in healthy BALB/c and tumor-bearing BALB/c mice. Curiously, when comparing healthy mice and 4T1-bearing mice, γδ-T cell biodistribution was similar in all tissues with the exception of the spleen where tumor-bearing mice had a significantly lower accumulation of γδ-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 γδ-T cells moderate the growth of mammary adenocarcinoma tumors.

Having established above that γδ-T cells do indeed localize to tumor, we next assessed to what extent adoptively-transferred γδ-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 γδ-T cells had a significant ($p = 0.02$) reduction in mammary tumor growth compared to un-

treated tumor-bearing mice. Importantly, during the course of these studies no untoward side effects were observed in mice treated with γδ-T cells.

Adoptive transfer of human γδ-T cells can moderate the growth of xenogeneic breast tumors.

To further assess the therapeutic potential of administering γδ-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 γδ-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 γδ-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 γδ-T cells.

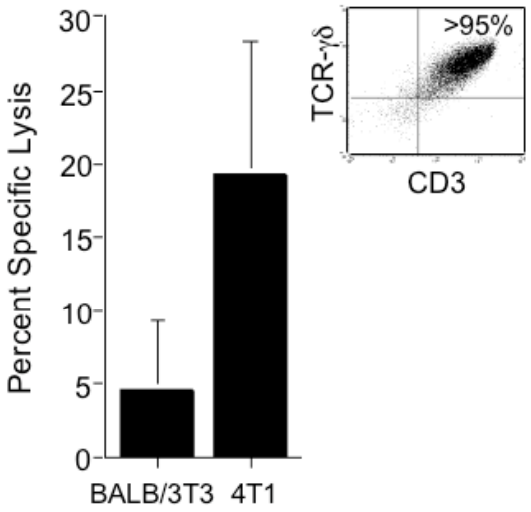

Figure 1. Ex vivo expanded BALB/c-derived γδ-T cells kill syngeneic 4T1 mammary adenocarcinoma cells but not syngeneic normal fibroblasts. γδ-T cells were obtained and expanded from the spleens of BALB/c TCR $\alpha\beta^{-/-}$ mice. FACS-plot inset shows representative purity of ex vivo expanded γδ-T cells employed in the in vitro cytotoxicity assays (and adoptive transfer studies). BALB/c-derived effector γδ-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 ± standard deviation. These results are from experiments performed three separate times.

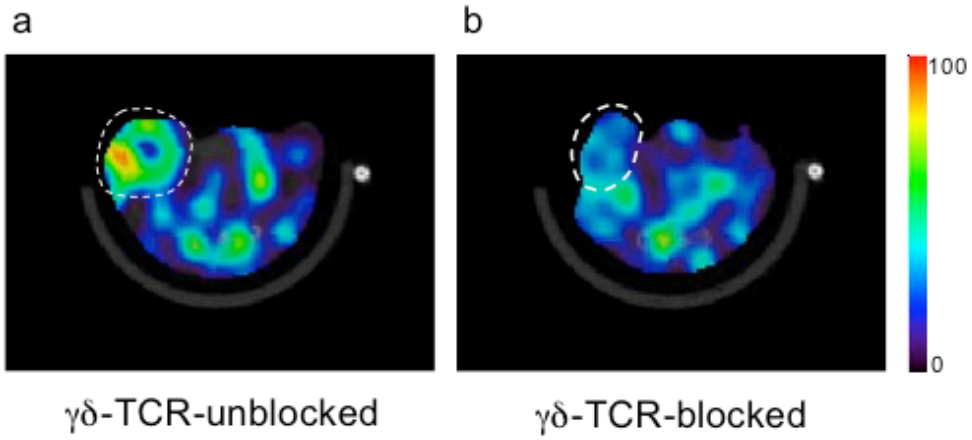

Figure 2**.** Adoptively-transferred γδ-T cells localize to 4T1 tumors. SPECT/CT fused images (axial view) showing distribution of ^{111}In-labeled γδ-T cells in 4T1 mammary fat pad tumors at 48 hours post injection. To demonstrate that the γδ-T cell receptor (γδ-TCR) itself is involved in the localization of adoptively-transferred cells to tumors, imaging was performed using ^{111}In-labeled γδ-T cells which were either (**a**) untreated or (**b**) pre-treated with an anti-γδ 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.

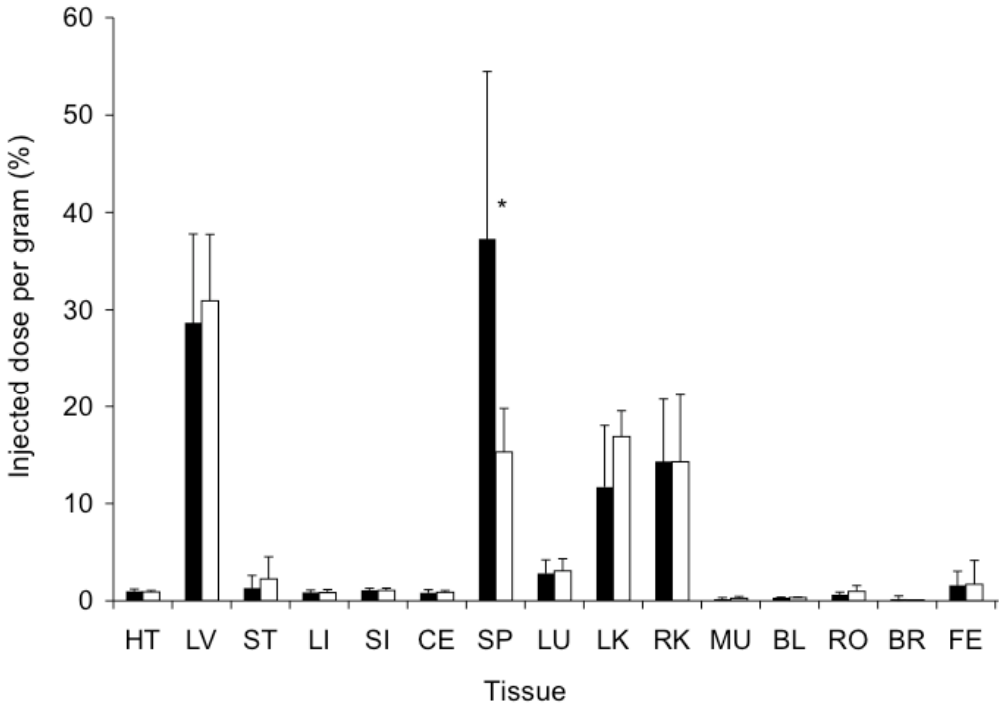

Figure 3. Adoptively-transferred γδ-T cells distribute differently within tumor-bearing mice as compared to healthy mice. ^{111}In-labeled γδ-T cells were injected into healthy mice or tumor-bearing mice. Biodistribution of administered γδ-T cells (expressed as a percent of injected radioactive dose 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. Statistically significant differences between tissues taken from healthy mice or from tumor-bearing mice are indicated by asterisks ($p = 0.003$ for spleen).

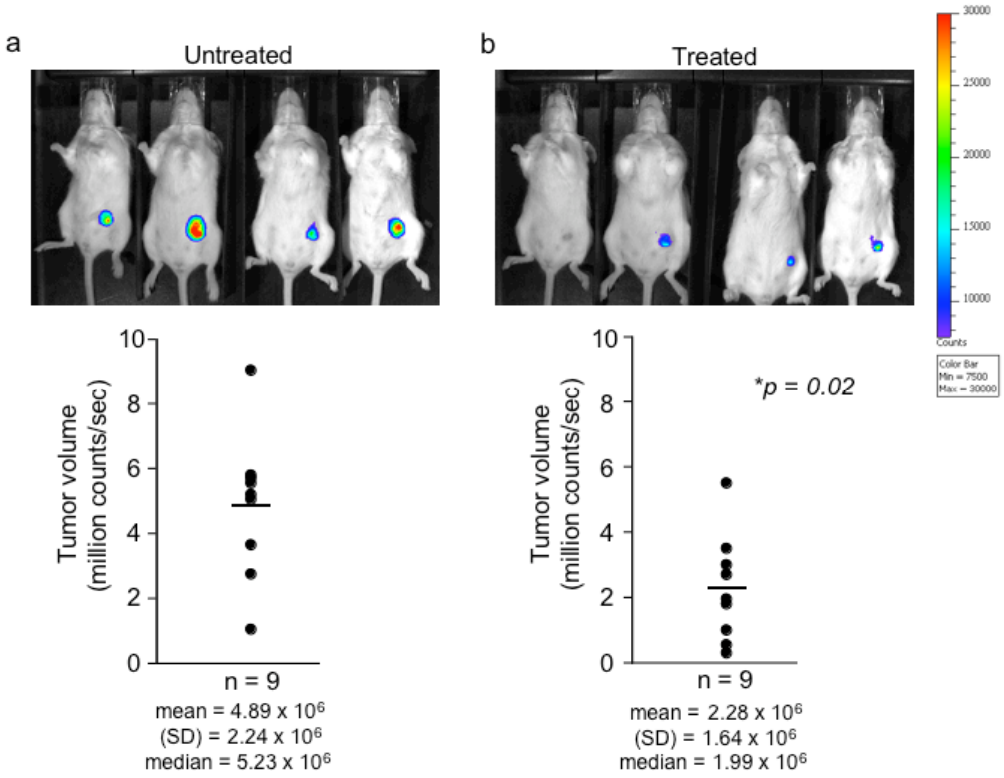

Figure 4. Bioluminescence studies: In vivo sensitivity of murine mammary adenocarcinoma cancer cell line 4T1-*Luc*2 to killing by adoptively-transferred syngeneic BALB/c γδ-T cells. 4T1-*Luc*2 cells were injected into the mammary fat pads of healthy female BALB/c mice. After 2 days, all animals were 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 γδ-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 (+/- 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 γδ-T cells is taken as evidence of in vivo γδ-T cell anti-tumor efficacy.

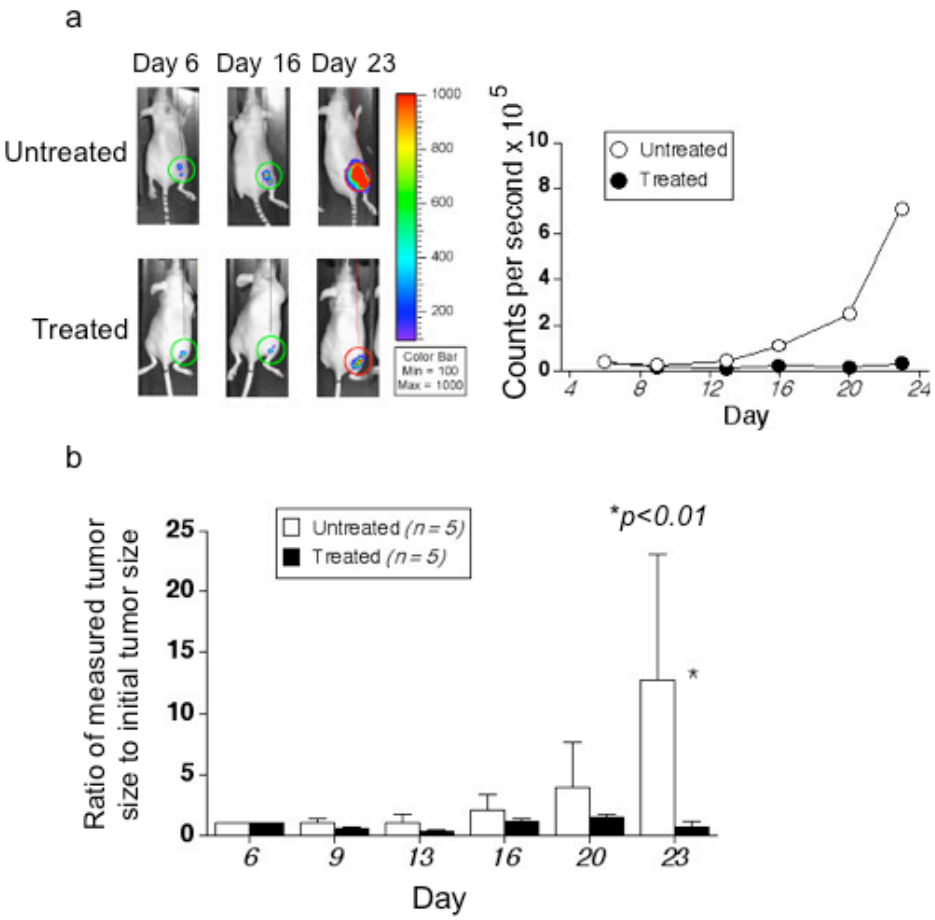

Figure 5. Bioluminescence studies: In vivo sensitivity of human breast cancer cell line 2LMP/*Luc* to killing by human γδ-T cells. Human breast cancer cell line 2LMP/*Luc* was derived by transducing 2LMP cells to express firefly luciferase using the recombinant adeno-associated virus-2 transduction methods [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 γδ-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 ± SD). Open bars, untreated animals (*n=5*). Solid bars, treated animals (*n=5*).

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, γδ-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 γδ-T cells [6, 12-15, 18]. Given the recognized capacity of γδ-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 γδ-T cells for clinical purposes [4, 10, 11, 17, 23, 28, 36-38, 42].

Although it remains to be determined how γδ-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 γδ-T cells within patients through the clinical administration of pharmacologic agents capable of stimulating human γδ-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 γδ-T cells may also be exploited through the adoptive transfer of γδ-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 γδ-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 γδ-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 γδ-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 γδ-T cells might best be administered therapeutically. Thus, we show that syngeneic BALB/c-derived γδ-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 γδ-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 γδ-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 γδ-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 γδ-T cells are first expanded ex vivo, then reinfused. In contrast to the alternative approach whereby endogenous γδ-T cells are activated in vivo within patients, in using the approach taken here, it becomes possible for investigators to experimentally track γδ-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 γδ-T cells (Figures 2 and 3), it will be possible to track therapeutically-administered human γδ-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 γδ-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 γδ-T cell-based immune therapies.

The observation that ^{111}In-labeled γδ-T cells readily localize to mammary tumors is not surprising (Figure 2), especially given our previous report that adoptively-transferred γδ-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-γδ-TCR antibody (mAb GL3) is consistent with our previous findings that in vitro, antibodies to the γδ-TCR only partially inhibit binding of γδ-T cells to sensi-

tive tumor cell targets [27]. In any event, the altered homing displayed by antibody-treated γδ-T cells could reflect disrupted trafficking, or alternatively, antibody-treated γδ-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 γδ-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 γδ-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 γδ-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 γδ-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 γδ-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 γδ-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 γδ-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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CANCER-DRIVEN EXHAUSTION OF TUMOR-REACTIVE γδ-T CELLS: RELEVANCE TO IMMUNOSURVEILLANCE AND IMMUNOTHERAPY

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ABSTRACT

We investigated a critical issue surrounding γδ-T cell-based cancer immunotherapy, which relates to the findings that γδ-T cells are numerically rare in the peripheral blood of some cancer patients, and only in some patients is it possible to activate and/or expand γδ-T cells either *in vivo* or *ex vivo* regardless of the methodology employed. This is in stark contrast to what is observed in healthy individuals, where γδ-T cells reliably respond to proliferative stimuli. Indeed, it is evident that such numerical or functional deficits may greatly hinder attempts to utilize γδ-T cells in clinical settings. Therefore, the objective of the present study was to examine the biological underpinnings that may explain the γδ-T cell impairments observed in cancer patients. To this end, we employed murine models of different malignancies (breast cancer, myeloma, and melanoma) and systematically examined γδ-T cell numbers in peripheral circulation, γδ-T cell proliferative capacity, and overall γδ-T cell condition in healthy mice and mice bearing different tumors. We found that tumor-bearing mice had fewer γδ-T cells in peripheral blood, and γδ-T cells derived from tumor-bearing mice expanded poorly ex vivo. We show that tumor cells were associated with the numerical and functional exhaustion of γδ-T cells. Using both *in vitro* and *in vivo* models of different cancers we demonstrated that γδ-T cells undergo apoptosis after encounter with tumor cells and we identified several putative genes involved in this exhaustion of γδ-T cells. Collectively, these findings will help facilitate the development of the next generation of clinical trials designed to exploit the tumor-reactive properties of γδ-T cells.

INTRODUCTION

Harnessing the tumor-reactive properties of the cellular immune system to treat malignancies is not a new concept. To date, most immune cell-based cancer immunotherapy strategies have focused on the stimulation of anti-tumor properties of the adaptive immune system such as with the vaccination of patients with tumor-specific antigens or antigenic peptides. Furthermore, while it may be feasible to identify and stimulate tumor-antigen-specific responses, particularly for malignancies with high immunogenicity such as melanoma, the tumor cell population simply does not remain static in the face of the anti-tumor force exerted by effectors such as αβ-T cells [10]. Thus, in due course immunologic pressures such as these may serve to edit or sculpt the immunogenicity of tumor cells and eventually lead to their complete escape from immunological attack [7]. Alternatively, both mouse and human γδ-T cells have been shown in numerous instances to possess the ability to directly recognize and kill malignantly transformed cells through less specific mechanisms that require no previous antigen exposure or priming (such as is required by the adaptive immune system).

Given the well-accepted capability of γδ-T cells to innately kill a variety of cancerous cells, efforts are now actively underway to develop and refine the means by which to exploit the antitumor properties of γδ-T cells for clinical purposes [3, 5, 6, 22, 35]. Currently, two specific approaches are being developed in this regard. One approach attempts the activation or expansion of the endogenous repertoire of γδ-T cells within patients through the clinical administration of pharmacologic agents capable of stimulating γδ-T cells, such as aminobisphosphonate drugs, typically in concert with cytokines such

as IL-2 [5, 6, 24, 32, 35]. In another approach, γδ-T cells are isolated from tumor-bearing patients, expanded ex vivo, and adoptively re-infused into the patient [22, 26].

While both approaches are clearly rational and will undoubtedly lead to significant advances in cancer treatment, an increasingly apparent issue to these approaches is emerging. This issue relates to γδ-T cells themselves found within patients. It was first reported that when compared to healthy donors, endogenous γδ-T cells are substantially fewer in numbers in the peripheral blood of newly diagnosed patients with certain cancers [1]. In our laboratory, we have confirmed and extended these clinical findings by documenting numerical deficits of γδ-T cells in the peripheral blood of glioblastoma, prostate, breast, and lung cancer patients (manuscripts in preparation).

Additionally, and perhaps more importantly, it appears that only in a proportion of patients is it possible to efficiently activate or expand patient-derived γδ-T cells either in vivo or ex vivo, which is a stark contrast to what is observed in normal, healthy individuals where robust activation or expansion is reliably achievable. More specifically, γδ-T cells derived from patients with melanoma or nasopharyngeal carcinoma (NPC) have been shown to expand poorly ex vivo [36] and in one study, patients with hematologic cancers exhibited impairments in γδ-T cell ex vivo proliferation so severe, it necessitated the "pre-screening" of study subjects for adequate in vitro γδ-T cell proliferation before their enrollment in clinical trials [35].

Indeed, it is evident that such numerical deficits may greatly hinder attempts to utilize either methodology designed to employ γδ-T cells in clinical settings. Therefore, the objective of the present study was to investigate the biological underpinnings that may explain the γδ-T cell impairments observed in cancer patients. To accomplish this,

we employed murine models of different malignancies (breast cancer, myeloma, and melanoma) and systematically examined γδ-T cell numbers in peripheral circulation, γδ-T cell proliferative capacity (ex vivo), and overall γδ-T cell condition in healthy mice and mice bearing different tumors.

Here, we show for the first time that cancerous cells are associated with the numerical and proliferative exhaustion of γδ-T cells. We demonstrate this using in vitro and in vivo models and determined that direct interactions with tumor cells, but not normal fibroblasts, causes the apoptotic death of γδ-T cells. Finally, using gene expression analyses we have identified several putative genes that may be involved in this process. These findings corroborate clinical reports and demonstrate that the numerical or functional failure of γδ-T cells observed in prior clinical studies is likely a tumor-driven process.

We propose that this cancer-associated γδ-T cell impairment could serve as a critical early event leading to impaired γδ-T cell anti-tumor immunosurveillance, which may contribute to the accelerated development or progression of different malignancies. Collectively, these findings will help facilitate the development of the next generation of clinical trials designed to exploit the tumor-reactive properties of γδ-T cells.

MATERIALS AND METHODS

Mice

C57BL/6 wild-type mice; BALB/c wild-type mice; C57BL/6 TCRβ-deficient mice ($TCR\beta^{-/-}$); and BALB/c TCR αβ-deficient ($TCR\alpha\beta^{-/-}$), mice were purchased from The Jackson Laboratory. Both male and female mice were used in experiments and were

7 to 16 weeks of age. All mice 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

The MOPC-315 plasmacytoma (H-2^d), BALB/3T3 normal fibroblast (H-2^d), 4T1 mammary adenocarcinoma (H-2^d), and B16F1 melanoma (H-2^b) cell lines were purchased from the American Type Culture Collection. Cells were maintained as recommended by ATCC.

Flow cytometry

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 BD Biosciences except where indicated: anti-CD3-APC (clone 145-2C11), anti-CD3-FITC (clone 145-2C11) anti-TCR-γδ-FITC (clone GL3), anti-TCR-γδ-PE (clone GL3), anti-CD16/CD32 (clone 2.4G2) anti-CD69-FITC (clone) and anti-Fas (clone).

Hematology

Whole blood was collected from the tail veins of healthy and tumor-bearing mice into tubes containing potassium-EDTA (BD Biosciences). Complete blood counts were determined using the Hemavet 950 (Drew Scientific). To calculate the absolute number of γδ-T cells/ml of peripheral blood, aliquots of whole blood were lysed with red blood

cell lysis buffer (eBiosciences). Cell preparations were washed once with PBS containing 3% FBS and stained for γδ-T cells using anti-γδ-FITC and anti-CD3-APC mAbs. The percentage of lymphocytes consisting of γδ-T cells was calculated by gating on the lymphocyte population (as determined by scatter properties). This percentage was used to determine the absolute number of γδ-T cells per ml of peripheral blood by multiplying by the absolute number of lymphocytes as determined with the Hemavet 950.

Preparation of ex vivo expanded mouse γδ-T cells

Ex vivo expansion of γδ-T cells was performed in studies designed to compare γδ-T cell expansion capability from healthy mice (BALB/c) and tumor-bearing mice (BALB/c with MOPC-315 or 4T1 tumors implanted) (Figure 1). In addition γδ-T cells were expanded ex vivo for use in cytotoxicity assays as previously described (Figure 5) [27]. Briefly, whole spleens were resected from mice ($TCR\alpha\beta^{-/-}$ for cytotoxicity assays; on either C57BL/6 or BALB/c background, whichever is syngeneic with target tumor cell line) homogenized, and then subjected to density gradient centrifugation using Lymphocyte Separation media (Mediatech). Cells were plated at a density of 5 X 10^6/ ml in RPMI-1640 containing 10% FBS, 2mmol/L-glutamine, 100 U/ml penicillin, 100 U/ml streptomycin, and 50 mM beta-mercaptoethanol in 6-well plates (Corning) coated with rat anti-mouse CD2 (5 mg/ml; clone RM2-5; BD Biosciences). Recombinant mouse IFN-γ (1000 U/ml; R&D Systems) and recombinant mouse IL-12 (10 U/ml; R&D Systems) were also added. After 24h, three volumes of fresh complete medium were added and cultures were stimulated with anti-CD3 mAb (clone 145-2C11; BD Biosciences) and 300

U/ml of recombinant mouse IL-2 (R&D Systems). Fresh medium along with 10 U/ml IL-2 was added every three days.

In vitro cytotoxicity assay

The cytotoxicity of ex vivo expanded C57BL/6 and BALB/c-derived γδ-T cells against different syngeneic cell lines was measured using the standard Chromium-51 release assay as described [28] or the CytoTox 96 Non-Radioactive Cytotoxicity Assay (Promega, Madison Wisconsin; this assay quantitatively measures lactate dehydrogenase (LDH), a stable cytosolic enzyme that is released upon cell lysis). γδ-T cells were harvested after eight days in culture (as described above) and were employed as effector cells at a 5:1 effector:target ratio against MOPC-315, BALB/3T3, 4T1, and at 10:1 against B16F1 target cell lines in a standard four hour co-culture incubation period.

Assessment of apoptosis

Cells were first surface stained using anti-CD3-APC and anti-TCR-γδ-PE mAbs. Cells were washed twice with cold PBS containing 3% fetal bovine serum, and stained for apoptosis using the TACS Annexin V-FITC Apoptosis Detection Kit (R & D Systems). Following Annexin V-FITC staining, cells were analyzed by flow cytometry. To confirm findings from Annexin V-FITC staining, cells were also stained using the Intracellular Caspase Detection Apostat kit from R & D Systems.

In vitro co-culture experiments

Spleens were resected from healthy BALB/c mice, homogenized, and red blood cells were lysed using red blood cell lysis buffer (eBiosciences). Splenocytes were cul-

tured in contact with MOPC-315, BALB/3T3, and 4T1, or separated by a 0.4 μm transwell insert that only allows for the exchange of soluble factors. Splenocytes were cultured at a 100:1 (splenocyte:cell line) ratio in complete media for 48 hours. After 48 hours, γδ-T cells were analyzed for apoptosis by FACS as described above.

In Vivo functional depletion of γδ-T cells

The hybridoma producing the anti-mouse-γδ-TCR antibody clone GL3 was kindly donated by Judith Kapp (University of Alabama at Birmingham) with permission from Leo Lefrancois (University of Connecticut Health Center). Two days before (day -2) and three days after (day +3) injection with 250,000 MOPC-315 cells, mice were administered 250 μg anti-γδ TCR mAb (clone GL3) intraperitoneally in sterile PBS or 250 μg of purified normal Armenian hamster IgG (Leinco Technologies).

Differential gene expression analyses

γδ-T cells were isolated from healthy BALB/c mice and mice bearing MOPC-315 tumors using fluorescence activated cell performed on a MoFlo (Dako Cytomation, Fort Collins, CO). The following antibodies were used for sorting and were obtained from BD Biosciences: anti-CD3-APC (clone 145-2C11), anti-TCR-γδ-FITC (clone GL3),and anti-CD16/CD32 (clone 2.4G2). Only living cells were sorted by gating the propidium iodide negative population. After sorting, γδ-T cells from healthy (sham-injected) BALB/c mice and MOPC-315-bearing BALB/c mice were pelleted and resuspended in 100 μl TRIzol (Invitrogen, Carlsbad, CA). Total RNA was extracted using the Arcturus PicoPure RNA Isolation Kit (MDS Analytical Technologies, Sunnyvale CA). The quality

of each RNA sample was determined by analysis on the 2100 Agilent Bioanalyzer prior to RNA labeling. Detailed genechip analysis procedures are presented in the Manufacturer's GeneChip Expression Technical Manual (Affymetrix). Briefly, 50 ng of total RNA from each sample was used in a two cycle cDNA amplification protocol using T7-linked oligo dT primers as per the manufacturer's instructions. After the first round of cDNA synthesis an *in vitro* transcription step was utilized to amplify the RNA and was followed by second round of cDNA synthesis. Subsequently, cRNA was generated and biotin was incorporated into the cRNA strand by standard methods (Affymetrix) followed by cRNA fragmentation, and preparation of hybridization cocktail. The arrays were hybridized overnight at 45^{o}C, and then washed, stained, and scanned the next day. Gene expression levels were extracted using AGCC (Affymetrix GeneChip Command Console). Statistical analysis of the GeneChip experiments were conducted using GeneSprings v10 (Agilent Technologies, Santa Clara, CA). Briefly, the raw GeneChip files from AGCC and Expression Console were uploaded, background-subtracted, variance stabilized, and normalized with GC-RMA method. The control group (healthy sham-injected mice) expression levels were used as a baseline to calculate the intensity ratio/fold changes of the treated (MOPC-315-bearing mice) versus the control group. The ratio was log2-transformed before further statistical analysis. The p-values are obtained by an unpaired t-test assuming unequal variance.

Statistical analyses

Comparison of means were performed using the two-tailed Student's *t* test. Comparisons include: the mean fold expansion of γδ-T cells derived from healthy BALB/c mice and

mice bearing either MOPC-315 or 4T1 tumors. The mean absolute number of γδ-T cells in healthy BALB/c and C57BL/6 mice and mice bearing MOPC-315, 4T1, or B16F1 tumors. The percentage of γδ-T cells undergoing apoptosis in vivo in healthy and tumor-bearing mice. The percentage of γδ-T cells undergoing apoptosis in vitro after co-culture with BALB/3T3, MOPC-315, or 4T1. The MOPC-315 disease burden in sham-injected and GL3 treated mice. To evaluate the relationship between MOPC-315 disease burden and apoptosis, a correlation was performed with an alpha level of 0.05 (two-tailed).

RESULTS

γδ-T cells derived from mice bearing tumors expand poorly ex vivo.

As reported, γδ-T cells in patients with hematologic malignancies are less responsive to both in vivo and ex vivo proliferative stimuli [24]. Moreover, γδ-T cells derived from patients with malignancies of epithelial origin also show similar evidence of impaired ex vivo expansion [1, 36]. Accordingly, we selected murine tumor models of both hematologic (MOPC-315) and epithelial (4T1) origin to comprehensively examine the extent to which mouse γδ-T cell expansion/proliferation may be affected by underlying malignant disease. Indeed, γδ-T cells exhibited poor ex vivo expansion when derived from the spleens of mice bearing either MOPC-315 ($p = 0.045$) or 4T1 ($p = 0.038$) and subjected to a published γδ-T cell expansion protocol [26] (Figure 1). Importantly, these studies were carried out in the absence of any treatment regimens such as chemotherapy and radiation; potential confounders of clinical studies that may contribute to meager γδ-T cell ex vivo expansion. We interpret these findings as early evidence that malignant disease may be a principle contributor to poor γδ-T cell ex vivo proliferation.

Mice bearing tumors have fewer γδ-T cells in peripheral blood.

We next examined γδ-T cells in mice bearing tumors in more extensive detail; in particular, we set out to more closely approximate the clinical situation. Accordingly, because peripheral blood is the accessible pool or source of γδ-T cells for ex vivo expansion in clinical studies, we initially attempted isolation of γδ-T cells from the blood of healthy mice and tumor-bearing mice. In doing so, we noticed a striking deficit in the numbers of γδ-T cells residing in tumor-bearing mouse blood, which precluded our ability to carry out the desired γδ-T cell expansions. This result was not surprising, as previous studies by others have indicated that patients with certain malignancies, such as melanoma, have decreased numbers of γδ-T cells in peripheral blood [1]. This led us to ask whether the tumor could be responsible for, or contribute to, this observed γδ-T cell numerical deficit. To examine this more thoroughly, we performed additional experiments using three murine models of different malignancies, myeloma (MOPC-315), mammary adenocarcinoma (4T1), and melanoma (B16F1). Ultimately, the purpose of these studies was to determine if the presence of tumors was associated with the numerical decline of γδ-T cells in the peripheral blood of mice. Consistent with the aforementioned clinical reports, the present animal studies revealed that in comparison to healthy age-matched BALB/c mice, mice bearing MOPC-315 and 4T1 tumors had significantly fewer ($p = 0.035$ and $p = 0.034$) numbers of γδ-T cells in their peripheral blood (Figure 2). Likewise, C57BL/6 mice bearing B16F1 melanoma tumors had far fewer ($p = 0.015$) γδ-T cells than age-matched healthy C57BL/6 mice in peripheral blood (Figure 2).

γδ-T cells in tumor-bearing mice are undergoing apoptosis.

To investigate the mechanism by which γδ-T cells may be lost in tumor-bearing mice we performed the following studies. In previous reports, groups have shown that γδ-T cells often undergo apoptosis after co-culture with various tumor cells [2, 8], however, whether this occurs in vivo has been unexplored. Thus, we revisited our in vivo tumor models with a particular emphasis on examining γδ-T cells for apoptosis. Accordingly, we established MOPC-315 and 4T1 tumors in BALB/c mice, after two to three weeks animals were euthanized, and γδ-T cells were stained for apoptosis using Annexin V-FITC and propidium iodide (viability) (Figure 3). From these studies we determined that mice bearing MOPC-315 and 4T1 tumors have a higher proportion of apoptotic γδ-T cells than healthy mice (Figure 3). However, in terms of apoptosis, αβ-T cells were un-affected by disease (Figure 3). Furthermore, using MOPC-315 (because of our ability to reliably quantitate tumor burden with an ELISA for M-protein) we show that as tumor burden increases γδ-T cell apoptosis correspondingly increases (Figure 3 *bottom*). Taken together, these data suggest that tumor cells may be associated with the numerical depletion or proliferative exhaustion of γδ-T cells through apoptosis.

γδ-T cell apoptosis is associated with tumor cell contact.

To more rigorously assess whether γδ-T cell apoptosis was a direct result of tumor, and not non-specific affects from the underlying disease, we performed the following series of studies. When tumor cells were co-cultured in vitro directly with splenocytes derived from healthy mice, a much higher percentage of γδ-T cells were undergoing apoptosis in comparison to when tumor cells were co-culture with splenocytes separated by a 0.4 μm transwell membrane (Figure 4). Furthermore, when normal fibroblasts were

co-cultured with splenocytes in contact or transwell, remarkably little γδ-T cell apoptosis was observed (Figure 4). Interestingly, as a positive control for apoptosis induction, the addition of conventional αβ-T cell mitogens (anti-CD3 and IL-2) to culture conditions for the same timeframe (48 h) resulted in marked γδ-T cell apoptosis [18]. We interpret these data as evidence that malignant cells are in some part responsible for the observed γδ-T cell apoptosis in tumor-bearing animals.

Syngeneic murine γδ- T cells exhibit in vitro cytolytic activity against tumor cell lines but not normal healthy cells.

Curiously, the interaction of γδ-T cells with tumor cell targets and purified tumor cell antigens has been shown to induce activation induced cell death in otherwise healthy cytotoxic γδ-T cells [2, 8]. It is well known that under certain conditions γδ-T cells are particularly sensitive to AICD and can readily undergo apoptosis upon activation [14, 17, 18, 28]. With these reports in mind, and our newest findings demonstrating that both a proliferative (Figure 1) and numerical (Figure 2) deficit exists in γδ-T cells within mice bearing tumors, we next asked whether γδ-T cells in our murine tumor models were diminishing as a result of cytotoxic interactions with tumor cells in vivo. The experiments here were designed to determine whether γδ-T cells isolated from healthy mice could *recognize* (as evidenced by killing) different syngeneic tumor cell lines. Reason being, if our γδ-T cell preparations were capable of exhibiting cytolytic activity against murine tumor cells, it would indicate that tumor cells could serve as a γδ-T cell mitogen stimulus in vivo. To examine this, we performed in vitro cytolytic assays that set in opposition our syngeneic γδ-T cells against either MOPC-315 (H-2^d, myeloma), BALB/3T3 (H-2^d, nor-

mal mouse fibroblasts), 4T1 (H-2^d, mammary adenocarcinoma), or B16F1 (C57BL/6 melanoma; data not shown, percent specific lysis at 10:1 γδ-T cell:tumor = 14.1%) (Figure 5). Indeed, BALB/c-derived (from BALB-TCR$\alpha\beta^{-/-}$) γδ-T cells were cytolytic against all malignant cells (Figure 5). However, γδ-T cells exhibited little cytotoxicity against non-malignant BALB/3T3 fibroblasts (Figure 5). Likewise, C57BL/6-derived γδ-T cells (from B6$\alpha\beta^{-/-}$) were cytolytic against B16F1 melanoma cells (data not shown). Importantly, these data show that γδ-T cells can distinguish between malignant and normal, healthy cells. From these data we propose that γδ-T cells recognize malignant cells—yet importantly, "ignore" normal healthy fibroblasts—the crucial activating mitogen stimulus and in due course γδ-T cells undergo apoptosis.

Experimental numerical depletion of γδ-T cells results in impaired anti-tumor immunosurveillance.

Next, to demonstrate the importance of having an intact (non-defective) γδ-T cell compartment, we set out to experimentally recapitulate the functional/numerical loss of γδ-T cells (as demonstrated in Figures 1 and 2) and subsequently challenge mice with tumor cells. We focused on MOPC-315 for this study because we can reliably quantify tumor burden using an ELISA specific for the myeloma protein produced by MOPC-315. Importantly, using γδ-T cell knockout mice, our laboratory has already demonstrated that the absence of γδ-T cells is permissive for the progression of murine prostate tumors [26]. Moreover, mice lacking γδ-T cells are more susceptible to the development of chemically-induced tumors and are less able to resist challenges with tumor cell lines [9, 11]. To deplete γδ-T cells, we injected mice with an anti-γδ-TCR monoclonal antibody (clone

GL3) or its corresponding isotype control (Armenian hamster IgG). After two days, mice were challenged with equivalent numbers of MOPC-315 cells. Twelve days later, the animals were sacrificed and MOPC-315 tumor burden was quantified by ELISA to detect the M315 protein produced by MOPC-315 (Figure 6). Figure 6 shows that mice depleted of γδ-T cells have a significantly greater tumor burden than mice with an intact (unmanipulated) γδ-T cell compartment. The experiments performed here confirm and extend the aforementioned reports supporting the view that γδ-T cells provide protective immunosurveillance against cancer. It is important to note here that these studies were not designed to simply add another tumor cell line to the growing list of those susceptible to γδ-T cell immunosurveillance pressures. Conversely, we sought to underscore the significance of the numerical deficits observed in tumor-bearing mice (as shown in Figure 2) by demonstrating the benefit of possessing a numerically and functionally intact γδ-T cell compartment.

γδ-T cells derived from tumor-bearing mice have upregulated genes involved in apoptosis and T cell activation.

The final objective of the current work was to compare gene expression profiles of highly purified γδ-T cells isolated from healthy BALB/c mice and tumor-bearing (MOPC-315) BALB/c mice. Gene expression levels were obtained using gene chip expression analyses (Affymetrix 430 2.0 arrays) and were confirmed using qRT-PCR. Select genes of interest found to be overexpressed in tumor-bearing mouse-derived γδ-T cells are shown in Table 1. Select genes include Aquaporin 1 (+5.1 log2). Intriguingly, the aquaporins are water channels that have been show to be expressed on thymocytes

and are absolutely critical for the initiation of apoptosis as they carry out the apoptotic volume decrease (AVD), an event whereby cells undergoing apoptosis first shrink to concentrate the cytosol for enhanced apoptotic enzyme function [16]. Lipocalin 2 (aka 24p3) was also overexpressed (+2 log2) in tumor-bearing mouse-derived γδ-T cells. Lipocalins are a diverse family of small, soluble proteins that are thought to augment cell death through various apoptotic properties [20]. Likewise, TGF-β was overexpressed (+3.1 log2) in diseased mouse γδ-T cells. Interestingly, splenic γδ-T cells isolated from tumor-bearing mice have previously been reported to transcribe mRNAs for TGF-β and were thought to represent an immunosuppressive subset of γδ-T cells [31]. Diseased mouse γδ-T cells also had greater expression of CCR5 a chemokine receptor frequently upregulated in activated T cells and previously shown to be expressed on human γδ-T cells [12]. An early marker of T cell activation CD69 was expressed also expressed at higher levels in γδ-T cells derived from tumor-bearing mice (+0.5 log2; confirmed with flow cytometry). It is important to note here, that these studies were not designed to compile an exhaustive genetic signature of exhausted γδ-T cells. In contrast, these studies were explicitly designed to confirm and extend our model that apoptosis/activation-induced cell death is the principle contributor to the impairment of γδ-T cells in tumor-bearing mice.

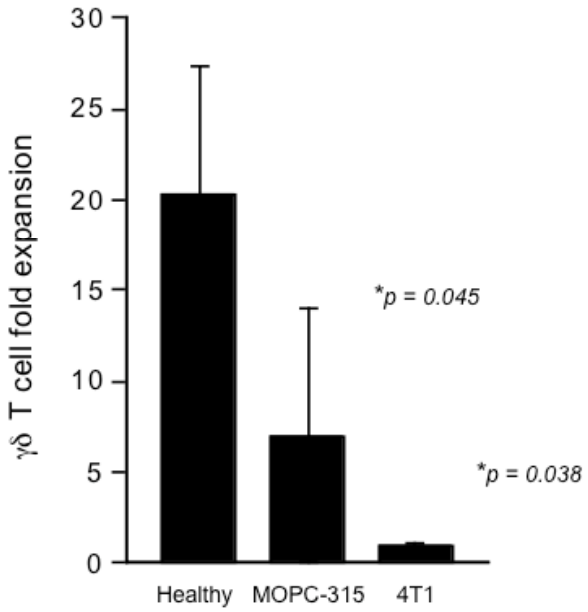

Figure 1. γδ-T cells derived from tumor-bearing mice expand poorly ex vivo. Mononuclear cells were obtained from whole spleens resected from either healthy BALB/c mice or BALB/c mice bearing MOPC-315 or 4T1 tumors. Cell preparations were cultured for 7 days using previously described expansion methods, after which fold-expansion of γδ-T cells was calculated from values obtained from cell counts (hemacytometer with Trypan Blue exclusion viability staining) and FACS analysis for γδ-T cells. Results are expressed as mean fold expansion of γδ-T cells, which was calculated by dividing the absolute number of γδ-T cells in cultures after 7 days by the absolute number of γδ-T cells before culture. Fold expansion was significantly lower in mice bearing MOPC-315 ($p = 0.045$) and 4T1 ($p = 0.038$) tumors in comparison to healthy BALB/c mice. Data are representative of at least three separate experiments for each group.

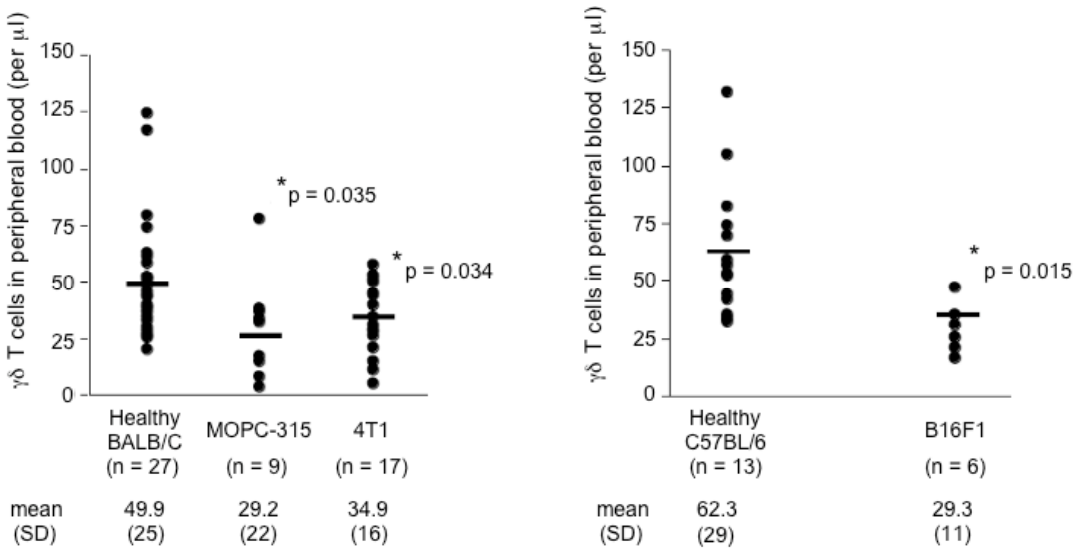

Figure 2. Tumor-bearing mice have fewer γδ-T cells in peripheral blood in comparison to healthy mice. Whole blood was isolated from the tail vein of healthy and tumor-bearing mice, and complete blood cell counts were obtained using a Hemavet 950 hematology analyzer. To determine the percentage of γδ-T cells in blood, PBMCs isolated from an aliquot of the whole blood were double-stained with anti-γδ-TCR-FITC (clone GL3) and anti-CD3-APC (clone 145-2C11) monoclonal antibodies, and analyzed with a FACSCalibur flow cytometer. (*Left*) The absolute number of γδ-T cells was significantly reduced in MOPC-315-bearing mice ($p = 0.035$) and 4T1-bearing mice ($p = 0.034$) when compared to age-matched healthy BALB/c mice. (*Right*) Similarly, mice bearing B16F1 tumors had significantly fewer ($p = 0.015$) γδ-T cells in their peripheral blood than healthy age-matched C57BL/6 mice.

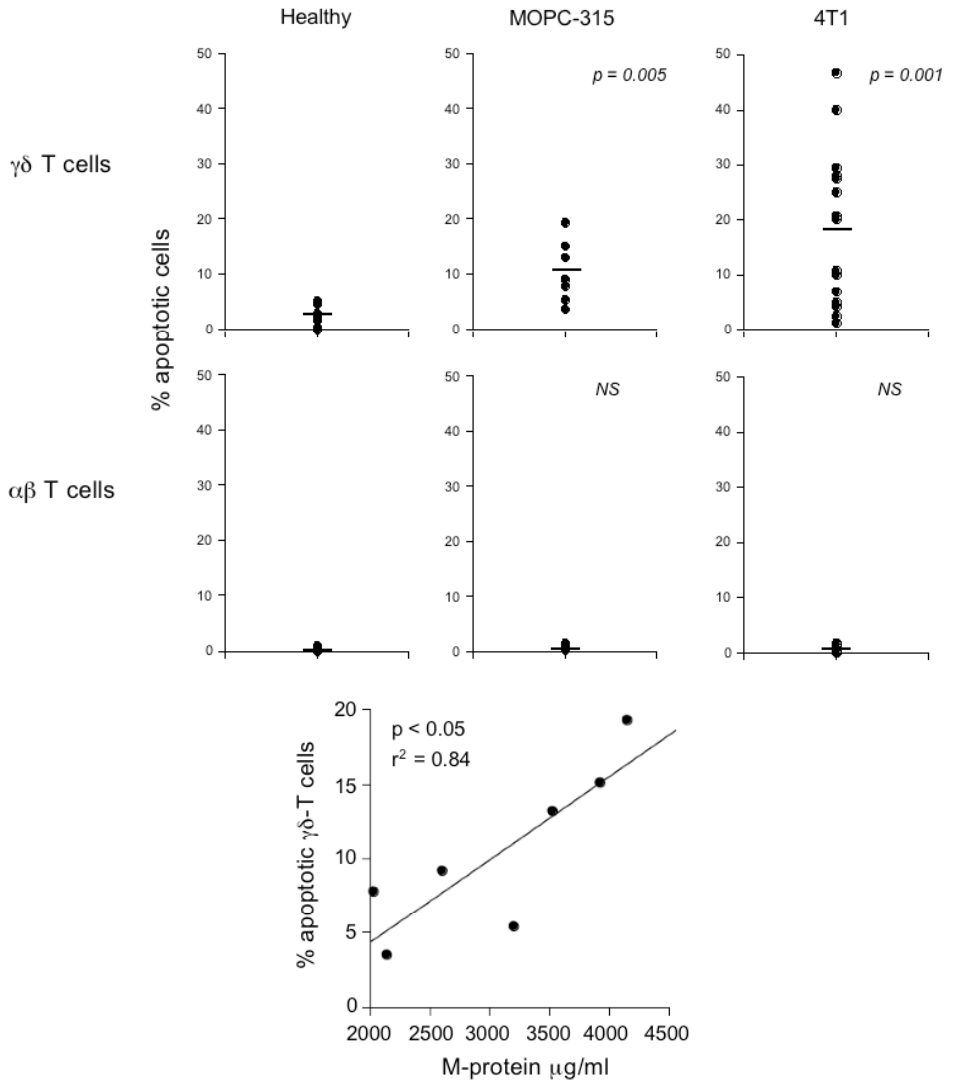

Figure 3. γδ-T cells in tumor-bearing mice are undergoing apoptosis. Eight-week to ten-week old BALB/c mice were used for these studies. Mice were injected intraperitoneally with 250,000 MOPC-315 cells or injected in the mammary fatpad with 40,000 4T1 cells and monitored for 14-19 days. Mice were sacrificed and PBMCs were isolated by Ficoll-Paque gradient centrifugation. γδ-T cells were analyzed for apoptosis by FACS. Viable γδ-T cells were identified by using anti-γδ-TCR-PE and anti-CD3-APC mAbs, and propidium iodide (viability). Apoptosis was determined by using Annexin V-FITC. A significantly greater percentage of γδ-T cells were apoptotic in mice bearing MOPC-315 ($p=0.005$) or 4T1 ($p=0.001$) tumors compared to healthy mice. There was no significant difference in αβ-T cell apoptosis between MOPC-315- or 4T1-bearing mice and healthy mice. (*lower panel*) When M-315 (MOPC-315 tumor burden) was measured in MOPC-315-bearing mice and compared to γδ-T cell apoptosis, there was a significant positive correlation ($R^2=0.84$; $p<0.05$) between tumor burden and γδ-T cell apoptosis.

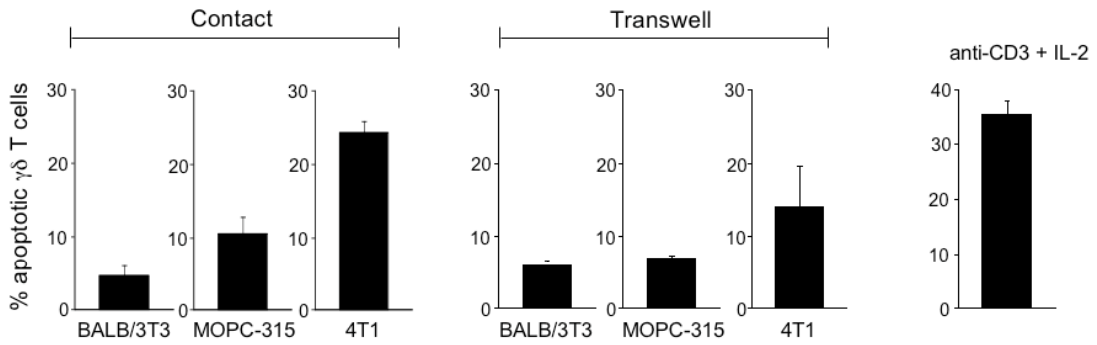

Figure 4. γδ-T cell apoptosis is tumor cell contact-associated. Combined data from four experiments are shown. Splenocytes derived from healthy BALB/c mice were cultured in contact with MOPC-315, BALB/3T3, and 4T1 or separated by a 0.4 μm transwell insert that only allows for the exchange of soluble factors. Splenocytes were cultured at a 100:1 (splenocyte:cell line) ratio in complete media for 48 hours. After 48 hours γδ-T cells were analyzed for apoptosis by FACS. Viable γδ-T cells were identified by using anti-γδ-TCR-PE and anti-CD3-APC mAbs, and propidium iodide (viability). Apoptosis was determined by using Annexin V-FITC. When splenocytes were cultured in contact with MOPC-315, 10.5% of γδ-T cells were undergoing apoptosis; however, when separated by a transwell membrane significantly ($p = 0.03$) less apoptosis (7.0%) was observed suggesting that tumor cell contact may be more important than tumor-derived soluble factors in contributing to γδ-T cell apoptosis. Similarly, when in contact, the 4T1 mammary adenocarcinoma cell line resulted in 24% of γδ-T cells undergoing apoptosis, while only 14% of γδ-T cells were apoptotic when tumor cells and splenocytes were separated by a Transwell insert ($p=0.038$). Furthermore, when splenocytes were cultured both in contact with, or separated from BALB/3T3, a nonmalignant cell line, there was significantly less apoptosis than when cultured in contact with MOPC-315 and 4T1.

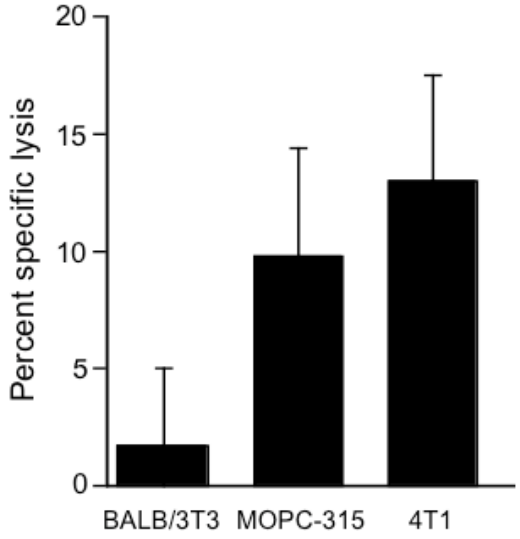

Figure 5. Ex vivo expanded γδ-T cells lyse syngeneic tumor cell lines but not normal fibroblasts. γδ-T cells were obtained and expanded from the spleens of BALB/c TCR $\alpha\beta^{-/-}$ mice. BALB/c-derived effector γδ-T cells were co-cultured with syngeneic target cell lines MOPC-315 (BALB/c myeloma), 4T1 (BALB/c mammary adenocarcinoma) and BALB/3T3 (normal BALB/c fibroblast cell line) and at a 5:1 effector:target ratio for 4 hours in a standard cytolytic assay. Specific cytotoxicity was determined and is expressed as percent specific lysis. Importantly, malignant cell lines MOPC-315 and 4T1 were readily killed by syngeneic γδ T cells, yet markedly reduced cytotoxicity was demonstrated when a normal, non-malignant cell line BALB/3T3 was the target; evidence that γδ T cells can distinguish between malignant and normal cell types.

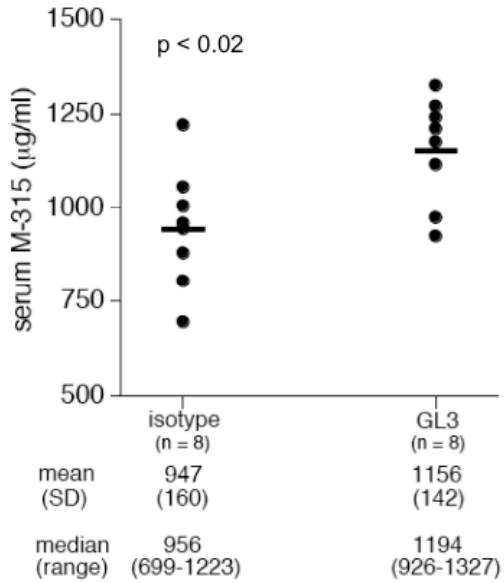

Figure 6. A defective γδ-T cell compartment is permissive for the progression of tumors. The experiments here were designed to experimentally mimic the numerical deficit observed in Figure 2. Eight to ten-week old wild-type BALB/c mice were used for these studies. Mice were depleted of γδ-T cells in vivo by treatment with GL3 antibody. Mice were injected intraperitoneally with either 250 μg of GL3 or isotype control two days before (day -2) and three days after (day +3) tumor cell injection. All mice were then injected intraperitoneally with 250,000 MOPC-315 tumor cells on experimental day 0. The M-315 myeloma protein produced by MOPC-315 cells was measured in serum samples using an ELISA. The serum M-protein concentrations (μg/ml) determined on day +12 in control mice (left) and γδ-T cell depleted mice (right) are shown. Mean M-protein concentrations (± SD) as well as median M-protein concentrations (with range) of the control group (n = 8) and the GL3-treated group (n = 8) are shown. Horizontal bars indicate the means of each group where $p<0.02$ when comparing the means (Student's t test). These data demonstrate that γδ-T cells play a significant role in the anti-tumor immunosurveillance in murine models of myeloma.

Gene (gene symbol)	Relative expression (log) over γδ-T cells from healthy mice	Putative role in exhausted γδ-T cells
Aquaporin 1 (Aqp1)	+5.1	Apoptosis
TGF-β	+3.2	Unknown
Lipocalin 2 (24p3)	+2	Apoptosis
CCR5	+1.2	Activation
CD69	+0.5	Activation

Table 1. Select genes putatively involved in the exhaustion of γδ-T cells within tumor-bearing mice. γδ-T cells were isolated from healthy BALB/c mice and mice bearing MOPC-315 tumors using fluorescence activated cell sorting. Gene expression levels were determined using an Affymetrix GeneChip (mouse 430 2.0 array). Gene expression levels were confirmed using quantitative PCR, except for CD69, which was confirmed at the protein level using flow cytometry.

DISCUSSION

Clearly, γδ-T cells are an important anti-tumor effector and their clinical exploitation may lead to significant advances in cancer treatment. However, despite early advances leading to the first generation of clinical trials utilizing γδ-T cells, we alongside others, have come to realize that a major obstacle exists which may preclude the complete utilization of these important cells, irrespective of the strategy employed (e.g., either endogenous expansion, or ex vivo expansion). This obstacle is the issue that γδ-T cells within patients appear to be damaged or exhausted.

For this reason, the studies undertaken in this report were performed with one principle objective in mind. We sought to examine the biological underpinnings that contribute to the exhaustion affecting γδ-T cells within tumor-bearing hosts, and in doing so, this work establishes for the first time that cancerous cells are associated with the in vivo demise of γδ-T cells.

Various mechanisms could contribute to the observed numerical and proliferative defects occurring in γδ-T cells isolated from either tumor-bearing mice or humans. Numerous reports have described a proliferative anergy of γδ-T cells, and proposed mechanisms for this anergy include: suppression of γδ-T cells by an overabundance of circulating tumor-derived antigens such as soluble MICA, dysregulated killer inhibitory receptor expression [21] (mostly CD94), and alterations in cytokine balance [4, 13, 15, 29]. More recently, one group has reported that $CD4^{+}CD25^{+}FoxP3^{+}$ regulatory T cells can specifically suppress γδ-T cells [25].

Moreover, several reports have established that γδ-T cells are exquisitely sensitive to apoptosis; importantly, conventional T cell mitogens (anti-CD3 and IL-2), purified tu-

mor cell antigens, and tumor cells have all been shown to induce apoptosis in γδ-T cells [2, 8, 18].

For the above reasons we developed a model to explain for the impairments of γδ-T cells within patients. Because γδ-T cells in vivo are repeatedly interacting with tumor cells, which express a variety of antigens that can be recognized by (and thus stimulate) reactive γδ-T cells, it stands to reason that repeated mitogenic stimulation eventually drives tumor-reactive γδ-T cells to undergo apoptosis. In theory, the induction of apoptosis and subsequent death of γδ-T cells is a logical explanation for the numerical losses of γδ-T cells observed in patients. Similarly, γδ-T cells that are actively undergoing apoptosis, would obviously display proliferative impairments.

To test our model, we first sought to determine if proliferative impairments were present in tumor-bearing animals. Indeed, γδ-T cells derived from tumor-bearing mice expanded poorly (Figure 1). Interestingly, these studies evolved from a previous study, where we demonstrated that adoptively-transferred BALB/c-derived (from *healthy* BALB/c mice) γδ-T cells were effective at controlling syngeneic (BALB/c) mammary tumors (4T1) (manuscript in preparation). However, in an effort to more explicitly model the clinical situation we attempted to derive γδ-T cells from tumor-bearing mice — in other words these mice were designed to represent "breast cancer patients" — from which, in a typical clinical setting, γδ-T cells would be expanded either endogenously or ex vivo. Importantly, our laboratory has previously identified a CD2-mediated, IL-12-dependent signaling pathway that inhibits apoptosis in mitogen-stimulated human peripheral blood-derived γδ-T cells [14, 28]. Currently, our laboratory favors this method for expanding human γδ-T cells. Thus, we utilized this method (using homologous mouse

reagents) to culture our γδ-T cells from healthy and tumor-bearing mice; however, as indicated in Figure 1, when derived from tumor-bearing mice the γδ-T cells were simply inert in terms of ex vivo proliferation. These findings supported our initial uncertainties about the condition of γδ-T cells within patients and we are currently investigating this issue further in hopes to develop an ex vivo expansion regimen that may serve to rescue γδ-T cells from this state of exhaustion.

As reported, in addition to exhibiting impaired γδ-T cell ex vivo expansion, a numerical deficit of γδ-T cells also exists in the peripheral blood of some cancer patients [1]. Importantly, from a clinical standpoint, peripheral blood is the source of γδ-T cells used for ex vivo expansion. Likewise, during endogenous γδ-T cell expansion protocols, γδ-T cell responses are monitored by examination of the peripheral blood from patients [35]. For these reasons, we deemed it imperative to examine the absolute numbers of peripheral blood γδ-T cells in tumor-bearing mice, as deficits in peripheral blood γδ-T cells would be problematic for either γδ-T cell immunotherapeutic methodology (ex vivo or endogenous). Indeed, in three separate models of malignancies (myeloma, breast, melanoma) and in two different mouse strains (BALB/c and C57BL/6), the circulating γδ-T cell compartment exhibited a marked numerical deficit (Figure 2). It stands to reason that the total systemic number of γδ-T cells could actually be similar in both healthy and tumor-bearing mice and that the numerical deficits of γδ-T cells observed here in tumor-bearing mice simply reflect altered trafficking. The most logical alternative location for the γδ-T cells "missing" from the peripheral blood of tumor-bearing mice, is within the tumor itself. Intriguingly, over a decade ago, one group reported that γδ-T cells rapidly

accumulate in early B16 melanoma lesions but after 5-7 days post-tumor injection, γδ-T cell numbers in tumor cell suspensions diminished greatly [30].

Collectively, we suspect that γδ-T cells in tumor-bearing mice are indeed "missing" altogether, and we propose a working model to explain this below. As evidenced in both MOPC-315 (myeloma) and 4T1 (breast cancer) syngeneic tumor models, a significantly greater number of γδ-T cells were apoptotic in mice bearing either tumor type (Figure 3). We also found a strong correlation between tumor burden and the proportion of γδ-T cells actively undergoing apoptosis, observing that animals with higher tumor burdens had in turn, a greater proportion of γδ-T cells actively undergoing apoptosis (Figure 3). Importantly, αβ-T cells examined in those same tumor-bearing animals were not undergoing apoptosis (Figure 3). Clearly, these findings do not establish that γδ-T cells are exclusively affected, as we did not examine an exhaustive list of other immune effectors for apoptosis. Curiously, a small proportion of γδ-T cells in healthy mice were undergoing apoptosis, which was not observed in αβ-T cells in healthy mice. We attribute this as a characteristic of γδ-T cell life history, as it has been reported that murine γδ-T cells display much higher turnover than αβ–T cells [34].

Despite our findings documenting marked γδ-T cell apoptosis in tumor-bearing mice, up to this point we had yet to establish that tumor cells, in any fashion, were directly responsible for the γδ-T cell apoptosis. Accordingly, we performed a series of in vitro studies to examine this. Indeed, Figure 4 clearly illustrates that a greater proportion of γδ-T cells are undergoing apoptosis when tumor cells are in co-culture with γδ-T cells than when separated by a 0.4 μm membrane. Perhaps of equal importance is the observation that remarkably little apoptosis is detected when a non-malignant cell line,

BALB/3T3 (normal BALB/c fibroblasts) is in co-culture or when separated by a membrane. Finally, as a positive control, when conventional T cell mitogens (anti-CD3 antibody and IL-2) known to induce activation-induced cell death in γδ-T cells were added to cultures, γδ-T cells underwent apoptosis. Of equal importance to these in vitro findings is illustrated in Figure 5 where we also show that γδ-T cells can lyse both MOPC-315 and 4T1 but fail to lyse BALB/3T3. Taken together (i.e., Figures 4 and 5), these findings provide strong evidence in support of our model where we propose that the exhaustion of γδ-T cells is a process dependent on the encounter with malignant cells. In other words, the same cell lines that are recognized and lysed by our γδ-T cells also induce contact-associated apoptosis.

The above data compelled us to perform another series of experiments in a different vein, more specifically, we set out to experimentally recapitulate the aforementioned numerical/functional defects in the γδ-T cell compartment to evaluate the consequences of a defective γδ-T cell compartment. For these experiments, we selected MOPC-315 because of the quantitative properties allowed by its M-protein production. Mice were first treated with a monoclonal antibody that has been shown to bind the γδ-TCR and subsequently inhibit or downmodulate (exact mechanism unknown) γδ-T cell function [19, 23]. Mice depleted of γδ-T cells developed much larger tumor burdens (as measured by serum M-315) compared to mice with an intact γδ-T cell compartment (Figure 6). It is well-known that mice lacking γδ-T cells are more susceptible to the development of tumors from chemical mutagens, spontaneously arising tumors, and challenges with various tumor cell lines [9, 11, 26, 33]. These findings are important for two reasons. First, these data confirm and extend the important findings of ourselves and others

by again demonstrating that γδ-T cells provide protective immunosurveillance against another tumor cell model. However, for our purposes, these findings are also important because they illustrate the consequences of a defective γδ-T cell compartment. Clearly, more investigation is needed into this issue, because as it stands these findings mimic the proverbial "chicken or the egg" argument. In other words, it stands to reason that certain individuals (or mice) could be predisposed to developing cancers by virtue of naturally possessing a numerically defective compartment. In contrast, individuals may indeed possess an intact γδ-T cell compartment but upon malignant transformation the γδ-T cell compartment may become exhausted.

The final objective of the current work was to compare gene expression profiles of highly purified γδ-T cells isolated from healthy BALB/c mice and tumor-bearing (MOPC-315) BALB/c mice. Indeed, in comparing gene expression levels we found that γδ-T cells from diseased mice overexpressed a few important genes with putative roles in apoptosis and T cell activation. While these data do not unequivocally identify a pathway contributing to the exhaustion of the γδ-T cell compartment, these data are crucial in supporting our working model that γδ-T cells within tumor-bearing mice are activated and displaying genetic characteristics of apoptosis. We realize that limitations to these data exist, particularly as gene expression arrays exclusively focus on RNA message, yet the biological effects presented here could result from biological fuctions such as post-translational modifications, splicing, etc.; all of which occur after transcription. Moreover, we limited our analysis to only the genes that exhibited marked differences in expression over control (healthy) mouse-derived γδ-T cells. It stands to reason, that genes

displaying subtle differences in expression could be of equal importance to those of heightened expression. We are currently investigating these data in further detail.

In summary, if activation-induced cell death is indeed accounting for the impairment of γδ-T cells in patients, strategies might be specifically developed to attempt to overcome this both *in vitro* and *in vivo*. Our laboratory is actively undertaking just such studies. Moreover, related to the immunobiology of γδ-T cell interactions with tumors, it will be key to gain a more clear understanding of what molecular targets are being recognized on tumor cells by γδ-T cells. Presuming these targets can be identified, new strategies might be developed to selectively or non-selectively modulate or upregulate these targets, thereby potentiating γδ-T cell-mediated killing of tumor cells and perhaps protecting against γδ-T cell impairment.

Nevertheless, despite the number of questions that remain, it is increasingly apparent that γδ-T cell-based immunotherapies hold great promise, and that the challenges identified here will be crucial in the design and implementation of future clinical trials intended to exploit γδ-T cells for their clinical purposes.

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CONCLUSIONS

Given the biological, technological, and pharmacological advances of recent years, human clinical trials intended to exploit the innate antitumor properties of γδ-T cells are now a reality [20, 21, 51, 83]. Yet, until the work performed in these dissertation studies, the view that tumor-reactive γδ-T cells could be used for the immunotherapy of breast cancer was supported only by a handful of *in vitro* studies designed to demonstrate cytolysis of human breast cancer cell lines by various γδ-T cell preparations [40, 59]. In this dissertation we show for the first time, preclinical in vivo mouse models of γδ-T cell-based immunotherapy directed against breast cancer. These studies were carefully designed to approximate clinical situations in which adoptively-transferred γδ-T cells would be employed therapeutically against breast cancer. Using radioisotope-labeled (^{111}In) γδ-T cells, we show that adoptively-transferred γδ-T cells do indeed localize to breast tumors in a mouse model of human breast cancer. Importantly, ^{111}In-labeling is a clinically applicable method which can be used to assess the trafficking patterns of adoptively-transferred T cells in patients [60]; however, until now, γδ-T cells have never been used in this context. By employing radio-labeled γδ-T cells, it becomes possible for investigators to experimentally track γδ-T cells after administration — a tool that will facilitate the design, interpretation, and refinement of future clinical trials.

Subsequently, and particularly important from a clinical perspective, we establish that ex vivo expanded γδ-T cells are not only effective against disease, but also do not cause untoward side effects upon adoptive transfer into tumor-bearing hosts. These

findings regarding efficacy and safety are paramount as clinical trials are being developed to assess how γδ-T cells might best be administered therapeutically.

As a whole, the findings described in the first chapter of this dissertation provide a strong biological rationale to justify the clinical use of adoptively-transferred γδ-T cells as a form of cancer immunotherapy for breast cancer. Indeed, we are preparing to embark upon our first generation of clinical trials. Accordingly, in the performance of such early phase trials, a more thorough understanding of the in vivo activity and fate of adoptively-transferred γδ-T cells will be key to refining later generations of trials. For this reason, the timeliness and relevance of our current findings are ideal, and will prove to be key in moving the field of γδ-T cell-based immunotherapy forward.

With the findings in mind from Chapter 1 of this dissertation, it would appear that γδ-T cell based immunotherapies are promising. Indeed, in my first days in the laboratory I mistakenly believed that irrespective of the malignancy, γδ-T cell-based immunotherapies were the solution. Unquestionably, in the latter chapter of the dissertation studies, we show γδ-T cells in a different light by essentially exposing a vulnerability of γδ-T cells. To be fair, the findings in Chapter 1 are critical in demonstrating the efficacy of γδ-T cell-based therapies. However, in our pursuit to more accurately model the clinical situation, we attempted to further these studies by using tumor-bearing mice as our source of γδ-T cells as opposed to healthy mouse donors.

From our findings in the second chapter of this dissertation, we put forth a model to explain the impairments within the γδ-T cell compartment in tumor-bearing hosts. We surmise that activation-induced cell death (via apoptosis) plays a major role in accounting for both the numerical deficits and the poor activation observed in γδ-T cells found in

tumor-bearing hosts. Indeed, it is well-known that under certain conditions γδ-T cells can be particularly sensitive to AICD and can quite readily be induced to undergo apoptosis upon activation [28, 39, 47, 48, 57, 73]. Accordingly, we propose that in the setting of cancer, γδ-T cells are lost as a consequence of AICD, this resulting from repeated encounter with tumor cells which express a variety of stress-induced self antigens which can be recognized by (and thus stimulate) reactive γδ-T cells. Consequently, it is this repeated mitogenic stimulation (i.e., AICD) which eventually drives tumor-reactive γδ-T cells to undergo apoptosis. This model is particularly appealing if one considers that tumor cells persist and thus remain as a source of chronic mitogenic stimulation for tumor-reactive γδ-T cells.

On the subject of future directions, we are undertaking two distinct but logically related strategies to advance γδ-T cell-based immunotherapies. First, and most obvious, we believe that the specific regimens used to expand γδ-T cells either endogeneously or ex vivo could be optimized to rescue γδ-T cells from exhaustion. Currently, we are investigating regimens that include the manipulation of the candidate target genes and their gene products identified in the studies above (i.e., Chapter 2; genes include aquaporin 1 (Aqp1), lipocalin 2 (24p3), and TGF-β). Furthermore, efforts are underway in our laboratory to coalesce the use of currently employed γδ-T cell expansion methods with other reagents and pharmacologic agents in hopes to rescue patient-derived γδ-T cells from an exhaustive state. As I am not permitted to go into detail here, we are currently evaluating a pharmacologic agent that has been shown to concomitantly activate T cells and protect them from apoptosis.

Alternatively, our second strategy is this: rather than relying upon potentially damaged or exhausted autologous (i.e., patient-derived) γδ-T cells for immunotherapy, we are taking an approach which allows us to adoptively transfer allogeneic (donor-derived) γδ-T cells obtained from healthy donors. We reason that tumor-reactive γδ-T cells obtained from healthy donors will be "undamaged" and in theory more effective. Moreover, donor-derived tumor-reactive γδ-T cells will be available in essentially limitless numbers from a healthy donor (after *ex vivo* expansion) and thus in theory can be delivered repeatedly to the tumor-bearing hosts.

However, the adoptive transfer of allogeneic γδ-T cells (or any T cell subset) into a tumor-bearing host is unlikely to be successful in the absence of specific immunological strategies undertaken to permit this. One such example strategy that we favor is allogeneic hematopoietic stem cell (HSC) transplantation. HSC transplantation continues to be a major curative treatment modality for various malignancies. One frequent component of a HSC transplant is the incorporation of post-transplant immunotherapy protocols, namely the donor lymphocyte infusion (DLI). In a DLI, mixed populations of lymphocytes are derived from the bone marrow donor and either infused with the marrow graft or at a later time-point. The purpose of the DLI is to lead to the development of an αβ-T cell mediated adaptive immune response in an effort to elicit anti-tumor activity. While this technique has proven to be highly valuable for the treatment and prevention of relapse in hematologic malignancies, limitations still exist in its inability to completely eradicate or lead to sustained remission in other malignancies. Equally important, severe risks are associated with a DLI, primarily in the form of graft-versus-host disease (GVHD).

In fact, GVHD continues to be a significant cause of morbidity and mortality after allogeneic HSCT. The pathogenesis of GVHD is complex, and GVHD can be classified as acute or chronic depending on the time of onset and clinical features. Interestingly, and pivotal to future therapies, γδ-T cells are thought to play an important role in the development of GVHD. In experimental animal models of allogeneic bone marrow transplantation, γδ-T cells have actually been shown to facilitate alloengraftment [23, 81] without causing GVHD [4, 24, 25]. Importantly, in humans, γδ-T cells have also been shown to potentially facilitate a graft-versus-leukemia effect, without causing GVHD, and that patients with increased numbers of γδ-T cells following an allogeneic HSCT have a survival advantage to those with fewer numbers of γδ-T cells [35]. Finally, γδ-T cells have been shown to provide protective immunity against numerous bacterial and protozoan pathogens; and as important, viral pathogens such as cytomegalovirus, a virus associated with significant transplant-related morbidity and mortality [10, 56, 82].

From the studies undertaken in this dissertation, it has become evident that γδ-T cell immunotherapies hold much promise, however, this promise does not come without challenges. Nevertheless, the future is bright for γδ-T cell-based immunotherapies as new areas of investigation have arisen from the identification of such challenges demonstrated in the present work. Indeed, clinicians and scientists now have the opportunity to extend the field in important ways allowing for the development of the next generation of clinical trials intended to make use of these fascinating cells.

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APPENDIX

IACUC FORM

THE UNIVERSITY OF ALABAMA AT BIRMINGHAM

Institutional Animal Care and Use Committee (IACUC)

NOTICE OF APPROVAL

DATE: December 26, 2007

TO: Richard D. Lopez, M.D.
WTI 558 3300
FAX: 934-1910

FROM: Judith A. Kapp, Ph.D., Chair
Institutional Animal Care and Use Committee

SUBJECT: Title: Innate Anti-Breast Cancer Activity of Gamma/Delta T-Cells: A Novel Biological and Clinical Approach to the Treatment of Relapsed or Refractory Breast Cancer
Sponsor: Department of Defense
Animal Project Number: 071207754

On December 26, 2007, the University of Alabama at Birmingham Institutional Animal Care and Use Committee (IACUC) reviewed the animal use proposed in the above referenced application. It approved the use of the following species and numbers of animals:

Species	Use Category	Number in Category
Mice	B	140

Animal use is scheduled for review one year from December 2007. Approval from the IACUC must be obtained before implementing any changes or modifications in the approved animal use.

Please keep this record for your files, and forward the attached letter to the appropriate granting agency.

Refer to Animal Protocol Number (APN) 071207754 when ordering animals or in any correspondence with the IACUC or Animal Resources Program (ARP) offices regarding this study. If you have concerns or questions regarding this notice, please call the IACUC office at 934-7692.

Institutional Animal Care and Use Committee
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