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Exploiting the Innate Antitumor Activity of Human γδ-TCells for the Treatment of Prostate Cancer

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14. ABSTRACT
We initially identified a CD2-mediated, interleukin (IL)-12–dependent signaling pathway which inhibits apoptosis in mitogen-stimulated human γδ-T cells. We have since exploited this pathway to develop the methodologies allowing the large-scale ex vivo expansion of viable apoptosis-resistant γδ-T cells. We have shown that apoptosis-resistant human γδ-T cells retain significant innate, major histocompatibility complex (MHC)-unrestricted cytotoxicity against human prostate cancer cell lines. **Purpose and scope:** The aims of this project have been, 1) to characterize the extent and breadth of the antitumor cytotoxicity mediated by apoptosis-resistant γδ-T cells against human prostate cancer cells; 2) to define the general mechanisms involved in the recognition and lysis of sensitive prostate cancer cells by apoptosis-resistant γδ-T cells; and 3) to determine the extent to which γδ-T cells can regulate the growth and metastasis of prostate cancer cells in vivo. **Key findings:** 1) Using the TRAMP transgenic mouse model of prostate cancer, we have formally demonstrated that absence of γδ-T cells is permissive for the development of tumors. 2) Conversely, we have shown that adoptively transferred mouse γδ-T cells are capable of producing the growth of syngeneic mouse prostate cancer cells (cell line TRAMP C2) in vivo and that in treated mice, γδ-T cells are shown to home to tumors in vivo. 3) γδ-T cell numbers are reduced in the peripheral blood of patients with prostate cancer; though it is not yet clear if this is related to the development or progression of disease. 4) We have shown that in vivo the in vitro capability of human γδ-T cells to kill human prostate cancer cells (PC-3) in xenografted nude mice.

15. SUBJECT TERMS
prostate cancer; immunotherapy; cellular therapy, gamma/delta T cell

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INTRODUCTION

We initially identified and characterized a CD2-mediated, interleukin (IL)-12–dependent signaling pathway which inhibits apoptosis in mitogen-stimulated human γδ-T cells. We have since exploited this pathway to develop the methodologies allowing the large-scale ex vivo expansion of viable apoptosis-resistant γδ-T cells – an undertaking until now, not possible. Importantly, we have shown that apoptosis-resistant human γδ-T cells retain significant innate, major histocompatibility complex (MHC)-unrestricted cytotoxicity against a wide variety of human-derived tumor cell lines, including human prostate cancer cell lines. This work has been reported, as noted below (Liu, Guo, Gehrs, Nan, and Lopez Journal of Urology 173:1552-1556, May 2005. See Appendix 1). Our efforts related to this proposal have remained focused upon testing the hypothesis that γδ-T cells – by virtue of their innate ability to recognize and kill epithelial-derived malignancies – play an important role in regulating the initial growth or spread of prostate cancer in vivo. The specific aims of this project have been, 1) to characterize the extent and breadth of the antitumor cytotoxicity mediated by apoptosis-resistant human γδ-T cells against human prostate cancer cells; 2) to define the general mechanisms involved in the recognition and lysis of sensitive prostate cancer cells by apoptosis-resistant γδ-T cells; and 3) to determine the extent to which apoptosis-resistant γδ-T cells can regulate the growth and metastasis of prostate cancer cells in vivo.

BODY

In the final report of this grant (spanning the period form April 1, 2003 to March 31, 2006), our accomplishments are presented in relation to the following tasks as outlined in the approved Statement of Work.

Task 1. To characterize the extent and breadth of the antitumor cytotoxicity mediated by apoptosis-resistant human γδ-T cells against human prostate cancer cells.

Initial objectives and rationale

The studies associated with this task are primarily observational (clinicopathologic correlations) and serve to establish the nature or magnitude of the defects in the γδ-T cell compartment of patients with prostate cancer.

Findings

1. Expansion, composition and antitumor activity of apoptosis-resistant human γδ-T cells: Healthy donors. It is important to point out that in healthy adult humans, fewer than 5 to 10% of circulating peripheral blood CD3+ T cells express the γδTCR and of these, ~ 60% express the Vδ2 TCR chain almost exclusively in combination with the Vγ9 TCR chain. The remainder of γδ-T cells express primarily the Vδ1 TCR chain in combination with a variety of other minor Vγ chains (1). As we have determined (2)(Figure 1, Appendix 1), the majority of apoptosis-resistant γδ-T cells which expand from peripheral blood mononuclear cells (PBMC) obtained from healthy donors express the Vδ2 TCR chain and not the Vδ1 TCR chain.

We have previously shown that ex vivo expanded apoptosis-resistant γδ-T cells retain significant innate antitumor activity against a wide variety of human tumor cell lines (3, 4), including now human prostate cancer cell lines DU-145 and PC-3 (2)(Figure 2, Appendix 1). Importantly, here we also show that γδ-T cells fail to kill normal human cells. In addition, we have determined that killing of prostate cancer cells by γδ-T cells can be blocked by mAbs to either the Vγ9 or the Vδ2 TCR indicating that the γδTCR is involved in the recognition and lysis of human prostate cancer cells (Figure 3, Appendix 1).

2. γδ-T cells in patients with prostate cancer. We have found that substantial differences appear to exist in the numbers of γδ-T cells present in the peripheral blood of patients with prostate cancer compared to
healthy donors. Importantly, these data are consistent with published data (as well as our own) where in other disease models – such as melanoma – a similar decrease in numbers of γδ-T cells are observed. Thus, when data are expressed as cells/µl for total γδ-T cells and for the Vδ1 and Vδ2 subsets (similar to how one would express a total CD4 helper T cell count), patients with prostate cancer have fewer total γδ-T cells compared to healthy donors. Total lymphocyte counts in patients and controls were not different suggesting that this a true loss in γδ-T cells, and not a non-specific lymphopenia. We do not yet know if these losses are a consequence of the existence of prostate cancer, or if these losses are permissive for the development of prostate cancer (see animal model findings below). These data are shown in Table 1.

After obtaining informed consent, 5 to 20 ml of whole blood was obtained from patients with prostate cancer or from healthy subjects. From each donor, a complete blood count (CBC) along with a differential cell count was obtained using a Coulter LH 750 analyzer. Aliquots of whole blood were stained for flow cytometric analysis using various monoclonal antibody combinations including FITC-Vδ1/PE-Vδ2/APC-CD3; FITC-γδTCR/APC-CD3; FITC-CD14/PE-CD3/APC-CD45; PE-CD20/APC-CD3 and the appropriate isotype control reagents. For flow cytometric analysis, lymphocyte gates included >98% of the CD3+ cells, >95% of the CD20+ cells, and <2% of the CD14+ cells. For each lymphocyte subset (i.e., total γδ-T cells, Vδ1+ γδ-T cells or Vδ2+ γδ-T cells) cell counts (expressed as cells/µl) were calculated by multiplying the whole blood lymphocyte count (obtained from Coulter counter) by the percentage of each given subset found within the lymphocyte gate as determined by flow cytometric analysis. Raw data for each donor are shown. The mean cell count for each subset (cells/µl ± SD) are also shown for healthy donors and for patients with prostate cancer.

Table I

γδ-T cell counts in healthy subjects compared to patients with prostate cancer

<table>
<thead>
<tr>
<th>Healthy Donors</th>
<th>Cell Counts</th>
<th>Vδ2 cells/µl</th>
<th>Vδ1 cells/µl</th>
</tr>
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<tbody>
<tr>
<td>A</td>
<td>TCR γδ cells/µl</td>
<td>55</td>
<td>26</td>
</tr>
<tr>
<td>B</td>
<td></td>
<td>75</td>
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<tr>
<td>C</td>
<td></td>
<td>115</td>
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<td>H</td>
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80* 66** 10***

(26) (23) (9)

<table>
<thead>
<tr>
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<th>Vδ2 cells/µl</th>
<th>Vδ1 cells/µl</th>
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</thead>
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<tr>
<td>POHU</td>
<td>68 y.o.; stage T1c, newly diagnosed, untreated</td>
<td>81</td>
<td>84</td>
</tr>
<tr>
<td>LYFR</td>
<td>76 y.o.; recurrence 13 yrs after initial therapy</td>
<td>13</td>
<td>9</td>
</tr>
<tr>
<td>ROED</td>
<td>69 y.o.; recurrence 4 yrs after initial therapy</td>
<td>36</td>
<td>18</td>
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<tr>
<td>HAGE</td>
<td>53 y.o.; stage T2c, newly diagnosed, untreated</td>
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<td>5</td>
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<tr>
<td>MAJO</td>
<td>69 y.o.; stage T1c, newly diagnosed, untreated</td>
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<td>CAMI</td>
<td>80 y.o.; stage T2a, on Lupron</td>
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<td>3</td>
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<tr>
<td>HIWA</td>
<td>70 y.o.; stage T1c, newly diagnosed, untreated</td>
<td>26*</td>
<td>22**</td>
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(27) (28) (6)

* P = 0.003 ** P = 0.017 *** NS
3. Expansion of γδ-T cells from patients with prostate cancer. Our studies to date indicate that apoptosis-resistant γδ-T cells can indeed be expanded from peripheral blood obtained from patients with prostate cancer. We have found that the absolute numbers of γδ-T cells expanding is somewhat less compared with healthy donors (not shown, analysis in progress). Importantly, ex vivo expanded γδ-T cells derived from patients with prostate cancer retain significant innate antitumor lytic activity in vitro against prostate cancer cell lines as shown in Figure 1.

![Figure 1](image.png)

**Figure 1.** Tumor-reactive γδ-T cells expanded from a patient with prostate cancer: Lytic activity demonstrated against prostate cancer cell lines: After obtaining informed consent, blood samples were obtained from an individual with newly diagnosed prostate cancer. As described, PBMC were prepared and expanded under protected conditions. After 14 days, γδ-T cells and αβ-T cells were isolated from cultures. Human prostate cancer cell lines DU-145 and PC-3 were first labeled with 51Cr. Purified γδ-T cells or control αβ-T cells were then incubated with tumor target cells at the indicated E:T ratios. After a 4 hr incubation, supernatants were removed to determine 51Cr release in CPM. Data are presented as the mean percent specific lysis of target cells (± SD) of triplicate determinations. In this instance, the subject was a 64 y.o. Caucasian man undergoing evaluation for an elevated PSA (11.9 ng/ml). On subsequent evaluation the patient was found to have adenocarcinoma of the prostate by biopsy (Gleason score 7) but no evidence of more extensive disease (stage T1c). At the time blood samples were obtained, the patient was untreated.

**On going work related to this task**

Establishment of primary cell lines from patient-derived samples. We have not successfully established primary tumor cell lines from patient-derived surgical specimens.

**Task 2. To define the general mechanisms involved in the recognition and lysis of sensitive prostate cancer cells by apoptosis-resistant γδ-T cells.**

**Findings**

The key findings related to this task are reported in a recently published manuscript (Appendix 1) These findings are that:

1. Monoclonal antibodies (mAb) against either the Vγ9 or Vδ2 T-cell receptor (TCR) chains.
2. In addition, mAb against intercellular adhesion molecules-1 (ICAM-1) or CD18 (β subunit of ICAM-1 counter-receptors) blocked γδ-T cell-mediated killing of prostate cancer cells.
3. γδ-T cells lysed prostate cancer cell lines largely through the perforin/granzyme pathway.
Task 3. To determine the extent to which apoptosis-resistant γδ-T cells can regulate the growth and metastasis of prostate cancer cells in vivo.

Initial Objectives and Rationale

These studies, as outlined in detail in the original proposal, were designed to use mouse models of prostate cancer to define the role of γδ-T cells in controlling the development and growth of prostate cancer. This has been accomplished by classical genetic means utilizing the TRAMP mouse model. In addition, xenograft studies have now been performed demonstrating the ability of human γδ-T cells to recognize and kill human prostate cancer cells which have first been established in mice. These findings are reported in more detail below.

Findings

1. Proof-of-principle studies: Absence of γδ-T cells is permissive for the development of prostate cancer. Utilizing the previously established TRAMP transgenic mouse model of prostate cancer (5, 6), we performed the initial studies shown in Figure 2 to determine the extent to which mouse γδ-T cells regulate the development or prevent the growth of prostate cancer in vivo. TRAMP mice were first backcrossed with mice lacking γδ-T cells (TCRδ−/−) or with mice lacking αβ-T cells (TCRβ−/−). Genotype of offspring was determined by PCR performed on tail DNA using the appropriate primers to detect the TRAMP transgene as well as primers for TCRδ. FACS analysis of peripheral blood using the appropriate antibodies to TCRγδ or TCRαβ was also used to identify the appropriate heterozygotes prior to additional rounds of breeding. Male mice of the selected genotype and were then used in these studies. Data shown in Figure 2 suggest that the absence of γδ-T cells is indeed permissive for the development of prostate adenocarcinoma.

![Figure 2. Absence of γδ-T cells is permissive for the development of cancer.](image)

Figure 2. Absence of γδ-T cells is permissive for the development of cancer. TRAMP mice are transgenic for a construct consisting of the minimal rat probasin promoter which drives expression of the SV40 early genes (T and t; Tag) in a prostate tissue-specific manner; TRAMP mice spontaneously develop prostate adenocarcinomas in a predictable manner. TRAMP mice were first backcrossed with commercially available TCRδ-chain knockout mice (TCRδ−/− mice, Jackson Labs, Bar Harbor, ME) which fail to develop γδ-T cells. A) Normal genitourinary (GU) tract of a male wild-type C57BL/6J mouse surgically removed at 7 months of age. B) GU tract of a male TRAMP mouse surgically removed at 7 months of age. Tumor infiltration of GU structures (including bladder, epididymis and prostate) were observed grossly and microscopically (not shown) in a manner consistent with published data. C) GU tract of a male TRAMP × TCRδ−/− animal removed at 5 months of age. Gross effacement of all GU structures was observed. Early sacrifice of TRAMP × TCRδ−/− animals was performed in accordance with animal welfare guidelines.
Data presented in Figure 3 show results of studies incorporating larger numbers of mice. This study shows that at age 7 months, TRAMP animals which lack γδ-T cells have larger tumor burdens compared to TRAMP animals (statistically significant difference). Interestingly, though not contradictory to our model, TRAMPβ−/− mice also appear to have more extensive prostate cancers compared to age-matched TRAMP mice. Whether γδ-T cells and αβ-T cells play a cooperative role in containing the development of adenocarcinoma in this model remains to be determined.

Representative data presented in Figure 4 (next page) further support the above findings. Here, tumor histology is presented along with a blindly determined histological grade of prostate tissues removed from control and experimental mice, as has been previously described (5, 7, 8). These data exclude the possibility that findings from Figure 2 and 3 represent a non-malignant hypertrophy of prostate tissues occurring in TRAMPδ−/− mice.

2. Adoptive Transfer of Syngeneic Mouse γδ-T cells Can Moderate the Growth of Syngeneic Mouse Prostate Cancer Cells In Vivo. TRAMP-C2 cells which were derived from TRAMP mice (and thus are of C57BL/6 origin) were used to establish syngeneic tumors in wild-type C57BL/6 mice. Figure 5 shows that the delivery of supraphysiological numbers of syngeneic C57BL/6 γδ-T cells into tumor-bearing animals can moderate the growth of C2-derived prostate cancer tumors in these mice. (Not shown: C2 cells are killed in vitro by syngeneic γδ-T cells in standard 51Cr-release assays). These data show in a statistically significant manner that adoptively transferred mouse γδ-T cells (in this case derived from TCRβ−/− mice which are on a C57BL/6 background) can substantially moderate the growth of syngeneic TRAMP-C2 tumors (C57BL/6 background) established in wild-type C57BL/6 mice.
Figure 4. TRAMP mice lacking γδ-T cells develop more aggressive prostate cancers compared to age-matched TRAMP mice with a normal γδ-T cell compartment. Histological analysis of prostate tissues. At 7 month of age, the GU tracts of mice were removed. Immediately upon sacrifice, all tissues were first fixed in formalin then embedded in paraffin. Sections cut from paraffin blocks (4–5 µm) were subsequently stained with hematoxylin and eosin (H&E) and examined by microscopy. Prostate lesions were scored blindly by an experienced pathologist using a 1–6 scale that has been established for TRAMP mice. Non-cancerous lesions were graded as 1 (normal tissue); 2 (low prostatic intraepithelial neoplasia, PIN); or 3 (high PIN). Cancerous lesions were graded as 4 (well-differentiated); 5 (moderately-differentiated); or 6 (poorly-differentiated). Representative histological images of prostates taken from C57BL/6 wild-type mice (left); TRAMP mice (center); and TRAMP γδ−/− mice (right) are shown here with the corresponding score indicated beneath.
Figure 5. Adoptively transferred mouse γδ-T cells moderate the growth of syngeneic mouse prostate cancer cells in vivo. Implantation of Tumor Cells and Randomization for Treatment. The TRAMP-C2 cell line (derived from TRAMP tumors which are of C57BL/6 origin) was used to establish tumors in healthy syngeneic C57BL/6 mice. Equivalent numbers of tumor cells ($3 \times 10^6$) were first injected subcutaneously into the thigh of each mouse. Fourteen days after tumor implantation, animals were randomly assigned to a treatment group or control group. Preparation of Ex Vivo Expanded Mouse γδ-T cells. Ex vivo expanded mouse γδ-T cells were prepared from spleens obtained from TCRβ−/− mouse. Spleen mononuclear cells were isolated by Ficoll-Paque gradient centrifugation. Similar to culture methods used for expanding human γδ-T cells, mouse γδ-T cell cultures were initiated at a cell density of $1 \times 10^6$ cells per ml in RPMI-1640 with 10% fetal bovine serum, 2 mmol/l L-glutamine, 100 U/ml penicillin, 100 U/ml streptomycin and 50 µM 2-ME. On the day of culture initiation (day 0), cells were transferred to tissue culture wells first coated with rat anti-mouse CD2 mAb clone RM2-5. Mouse recombinant interferon-γ (1,000 U/ml) and recombinant mouse IL-12 (10 U/ml) were then added at the indicated final concentrations. After 24 hours (day 1), cultures were stimulated with 10 ng/ml anti-CD3 mAb clone 145-2C11 (hamster, IgG1) and 300 U/ml mouse recombinant IL-2. Fresh medium with 2 U/ml IL-2 was added every 3 days. At day 8, the cells were harvested. Purity of γδ-T cells was assessed by FACS and was routinely found to be greater than 95% with greater than 90% viability. Treatment of Tumor-bearing Mice and Assessment of Response. On experimental days 14, 19, 21, 26 and 28, γδ-T cells ($20 \times 10^6$ cells per treatment) were administered IV by tail vein injection into tumor-bearing animals (treated, n=7, right A). Control animals (untreated, n=7, left A) received only PBS injections on the same days. Tumor sizes in all animals was assessed periodically by calculating tumor weight based upon two-dimensional measurements using established methods where calculated tumor weight (mg) = [Width (mm)$^2$ × Length (mm)] / 2. Tumor size data for every individual mouse in both the treated and untreated groups are plotted above in A. Bar graph in B presents these same data as the mean tumor size (mg ± STD) of the treated group (n=7, filled bars) and the untreated group (n=7, open bars). P values were calculated using the Student's t-test.
3. Studies to Examine the Localization of Adoptively Transferred γδ-T cells into Tumors. Green fluorescence protein (GFP)+ γδ-T cells were derived from cultures of GFP × TCRβ−/− mouse spleens. Data in Figure 6 indicate that green fluorescence (GFP)+ γδ-T cells can be detected by fluorescence microscopy within tumor tissues – this strongly suggesting that adoptively transferred γδ-T cells can localize into tumor tissues. This is shown in A (left) where frozen sections are examined and in B (right) where fresh tissues are examined. Importantly, in each case, GFP+ cells are not seen within samples of normal muscle tissue excised from tumor-bearing mice, suggesting that adoptively transferred γδ-T cells are indeed localizing preferentially into tumors or into tissues surrounding tumors.

![Figure 6. Detection of adoptively transferred GFP+ γδ-T cells in tumor tissue but not normal tissue. A) Tumor-bearing nude mice: Frozen sections. Tumor-bearing nude mice which were treated with GFP+ γδ-T cells were sacrificed shortly after blood sampling performed on day 6. Immediately upon sacrifice, tumors were excised then rinsed with PBS before being frozen. Similarly, normal muscle tissue was excised from the opposite hind leg of the same treated animals. Both tumor tissue and control muscle tissue were sectioned into approximately 27 mm³ samples. As a control for non-specific background fluorescence, tumor tissue was also excised from mice that received no GFP+ γδ-T cells (right, untreated). Frozen sections were examined directly using a Nikon Eclipse TE 2000-U inverted fluorescence microscope fit with a CoolSNAPES digital camera (Photometrics Inc). Images shown here are magnified 200 times and are presented as pseudo-color images derived using IPLab image processing software (version 3.9.2 for Mac OS X, Scanalytics, Inc.). B) Tumor-bearing wild-type C57BL/6 mice: Fresh tissues. Tumor-bearing wild-type C57BL/6 mice which were treated with GFP+ γδ-T cells were sacrificed shortly after blood sampling performed on day 8. Immediately upon sacrifice, tumors were excised then rinsed with PBS, but not frozen. Normal muscle tissue was excised from the opposite hind leg of the same treated animal, but not frozen. As a control for non-specific background fluorescence, tumor tissue was also excised from mice that received no GFP+ γδ-T cells (right, untreated). Whole fresh tumor specimens or whole muscle sections were then examined directly by fluorescence microscopy, as described above. Images shown here are magnified 100 times.](image)

4. Establishment of an in vivo bioluminescence model for the immunotherapy of murine syngeneic C2 prostate cancer: Figure 7 (next page) shows that we have been able to successfully establish a new cell line (C2.RFP) which is cell line C2 transfected to express red fluorescence protein (RFP). The potential importance of this achievement is underscored when one considers that when optimized, in vivo bioluminescence techniques can detect as few as several thousand viable tumor cells. This gives us the ability to treat and assess tumor-bearing animals which have truly minimal disease. Such a model will be extremely useful in the development of γδ-T cell-based immunotherapies for minimal residual disease, including post-surgical models of local treatment failure. Moreover, using green fluorescence protein (GFP)-expressing γδ-T cells (generated by backcrossing), we also now have the ability to examine the homing of tumor-reactive γδ-T cells to both primary and metastatic sites of tumor. These and related models will be developed in future studies.
A) Generation of cell lines detectable by in vivo bioluminescence. **Tumor target cells (TRAMP-C2.RFP):** Red fluorescence protein (RFP)-expressing mouse prostate cancer cell line TRAMP-C2.RFP was generated by transfecting TRAMP-C2 cells with pCMV-DsRed-Express vector (BD Biosciences). Transfection was accomplished utilizing a polyfect transfection reagent kit following the manufacturer's recommended protocol (Qiagen). Forty eight hours after transfection, cells were split and cultured under G-418 selection. Cells were kept under stringent selection pressure for one month and subsequently subjected to single-cell sorting (FACS DiVa flow cytometer, Becton Dickinson) followed by further culture under less stringent G-418 selection. Clones were subsequently selected and expanded. A representative RFP-expressing TRAMP-C2 clone is shown here under fluorescence microscopy using the appropriate excitation and emission filters for visualization of RFP. **C57BL/6 (syngeneic) cytolytic effector cells (GFP+ γδ-T cells):** Utilizing immunomagnetic separation methods, green fluorescence protein (GFP)-expressing γδ-T cells were isolated directly from mouse spleen cell preparations derived from commercially available mice which are transgenic for GFP (C57BL/6 background, Jackson Labs). Alternatively, mice transgenic for GFP were first crossed with commercially available mice lacking αβ-T cells (TCRβ−/− mice on the C57BL/6 background, Jackson Labs). After appropriate screening and backcrossing, a colony of GFP-transgenic animals lacking αβ-T cells was established. As these animals lack contaminating αβ-T cells, extremely pure populations of GFP-expressing γδ-T cells are readily obtained for further purification or expansion. Freshly isolated GFP-expressing γδ-T cells are shown here under fluorescence microscopy using the appropriate excitation and emission filters for visualization of GFP. B) C57BL/6 GFP+ γδ-T cells lyse syngeneic mouse TRAMP-C2.RFP cells. TRAMP-C2.RFP cells were first labeled with 51Cr. GFP+ γδ-T cells or control GFP+ αβ-T cells were isolated by immunomagnetic column from spleen cell preparations derived from GFP-transgenic C57BL/6 mice. 51Cr-labeled TRAMP-C2.RFP target cells were incubated (E:T ratio = 10:1) with effector GFP+ γδ-T cells (solid bars) or GFP+ αβ-T cells (open bars). After a 4 hr incubation at 37° C, supernatants were removed to determine 51Cr release in CPM. Data are presented as the mean percent specific target lysis (± SD) of triplicate determinations. C) In vivo bioluminescence image of wild-type C57BL/6 mice bearing syngeneic TRAMP-C2.RFP-derived tumors. TRAMP-C2.RFP cells (1 × 106) were first introduced subcutaneously into the flank of each of 2 separate wild-type C57BL/6 mice. Animals were immediately imaged to document delivery of viable tumor cells (not shown). After 21 days, both animals were re-imaged to quantify detectable tumor as determined by light emission in photons per second from each region of interest analyzed (green circles). Whole animal images were obtained on an IVIS Imaging System Series 100 bioluminescence detector. Light emission (using the appropriate filters for RFP) from each region of interest is represented as a pseudo-color scaling superimposed upon black-and-white images obtained concurrently.
5. **In vivo sensitivity of human prostate cancer cell line PC-3 to human γδ-T cells: A pre-clinical xenograft model for γδ-T cell-based therapies for prostate cancer.** Data in **Figure 8** show that the human γδ-T cells – when delivered intravenously into mice harboring human prostate cancer cells – can substantially moderate the growth of these tumors. These findings are essential to our further development of γδ-T cell-based models for the adoptive cellular therapy of human prostate cancer.

![Figure 8](image_url)

**Figure 8. In vivo sensitivity of human prostate cancer cell line PC-3 to human γδ-T cells: Mouse xenograft model.** Human prostate cancer cell line PC-3 was used to establish tumors in nude mice. Equivalent numbers of tumor cells (0.5 × 10^7) were first injected subcutaneously into age-matched and sex-matched animals. Beginning one week after tumor implantation, individual animals were treated with ex vivo expanded human γδ-T cells derived from separate healthy donors. Cells were administered intravenously on days 9, 14, 19 and 24 to animals in the treated group. Cells dosed ranged from 10 to 20 × 10^6 cells delivered per treatment. Control animals were left untreated and received only PBS. Tumor growth was assessed periodically in all animals by calculating tumor weight based upon two-dimensional measurements using established methods. Results are expressed as the mean tumor weight (mg ± standard deviation) of treated animals (filled circles, n=3) and control animals (open circles, n=5).

**KEY RESEARCH ACCOMPLISHMENTS**

- We can now conclude that ex-vivo expanded human Vγ9Vδ2+ γδ-T cells are able to innately recognize and kill certain human prostate tumor cell lines in vitro. Recognition and killing of prostate cancer cells occurs in a γδ-TCR–dependent manner and also appears to involve adhesion occurring through ICAM-1 and CD18. The cytolytic process involves primarily the perforin/granzyme mediated pathway of granule exocytosis. These results are now published (see Appendix 1).

- Accumulating data suggest that there exists a deficit in the γδ-T cell compartment of patients with prostate cancer. Whether this deficit is a result of the presence of cancer, or is permissive for the development or progression of cancer is not known. This is actively under investigation.

- We have validated and confirmed key proof of principle studies in our animal model which support our view that the absence of γδ-T cells is indeed permissive for the progression of prostate cancer. These studies support our overall hypothesis.

- We have shown that adoptively transferred mouse γδ-T cells can moderate the growth of syngeneic mouse prostate cancer cells in vivo (in vivo models for the adoptive immunotherapy of prostate cancer).

- We have demonstrated in vivo that the growth of human prostate cancer cell-derived tumors can be moderated by human γδ-T cells. These findings are directly relevant to our overall goals of developing the means to exploit the innate antitumor activity of γδ-T cells for the immunotherapy of prostate cancer.
REPORTABLE OUTCOMES

Publications


*Note: Findings reported on pages 4-13 are currently being prepared for submission for publication.*

New Funded Grants

Title: Clinical-scale Expansion and Purification of Human γδ-T cells for the Adoptive Cellular Therapy of Advanced or Recurrent Prostate Cancer
Agency: Prostate Cancer Foundation
Type: Research Award
PI: Richard Lopez

The studies outlined in this recently funded award are specifically intended to integrate the necessary biological, clinical and regulatory issues in order to efficiently secure FDA approval for our first generation investigator-initiated clinical trials. Thus, the support provided by the Prostate Cancer Foundation will serve as the crucial bridge between our pre-clinical experimental models funded by the DoD (i.e., in vitro and animal-based studies) and the successful design and execution of our first clinical trials to be funded with future grants intended to support such trials. Please see *Appendix 2* which is an excerpt from the awarded grant.

Grants to be submitted or under review

Title: Innate antitumor immunity of γδ-T cells: New models for targeting prostate cancer
Agency: NCI
Type: R01
P.I.: Richard Lopez, M.D.
Submit: June, 2006

Presentations

Second International Conference on γδ-T cells
Scripps Institute, February 22-28, 2006, San Diego, CA
"Role of γδ-T cells in the containment or eradication of prostate cancer" (R. Lopez).

Duke University Bone Marrow Transplant Program Grand Rounds, September 13, 2005, Durham, NC
"Innate Anti-tumor and Anti-viral Immunity of γδ-T cells: Biological and Clinical Models for the Adoptive Immunotherapy of Malignant and Infectious Diseases" (R. Lopez)

Emory University Winship Cancer Institute Hematology Grand Rounds, February 2, 2006, Atlanta, GA
"Negotiating the biological, clinical and regulatory obstacles to modern cell-based immunotherapy: The ongoing γδ-T cell experience" (R. Lopez)
CONCLUSIONS

• As noted above, the findings that we report here are support our overall hypothesis that, "By virtue of their ability to innately recognize and kill epithelial-derived malignancies, γδ-T cells play an important role in regulating the initial growth or spread of prostate cancer in vivo".

• As importantly, our latest findings support our prediction (made in relation to our overall hypothesis, as stated in the original grant) that, "The large-scale ex vivo expansion of apoptosis-resistant human γδ-T cells will allow for the direct clinical administration of cells possessing innate antitumor activity against prostate cancer. In conjunction with additional therapies, this and related approaches will redefine the future treatment of recurrent or metastatic prostate cancer". As noted above, the studies performed as part of this grant have been instrumental in securing additional funding (Prostate Cancer Foundation) specifically intended to develop human clinical trials in which we intend to deliver human γδ-T cells to patients with prostate cancer.

REFERENCES


APPENDICES

• Published manuscript attached
• Prostate Cancer Foundation Grant (funded 1/2006)
EX VIVO EXPANDED HUMAN Vγ9Vδ2+ γδ-T CELLS MEDIATE INNATE ANTI-TUMOR ACTIVITY AGAINST HUMAN PROSTATE CANCER CELLS IN VITRO

ZHIYONG LIU, BEN L. GUO, BRADLEY C. GEHRS, LI NAN AND RICHARD D. LOPEZ*

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ABSTRACT

Purpose: We have previously identified a CD2 mediated, interleukin-12 dependent signaling pathway that inhibits activation induced cell death in mitogen stimulated human γδ-T cells, permitting the large-scale expansion of these cells. Herein we report the innate antitumor activity of expanded human Vγ9Vδ2+ γδ-T cells against human prostate cancer cells.

Materials and Methods: Apoptosis resistant human γδ-T cells were expanded in vitro from cultured human peripheral blood mononuclear cells and then enriched to high purity by immunomagnetic separation. In vitro cytotoxicity of expanded γδ-T cells was measured against human prostate cancer cell lines using standard cytotoxicity assays.

Results: γδ-T cells derived from various donors consistently showed lytic activity against the prostate cancer cell lines DU-145 and PC-3 but not LNCaP. mAbs against Vγ9 or Vδ2 T-cell receptor chains as well as mAb against intercellular adhesion molecule-1 (ICAM-1) or CD18, the β subunit of ICAM-1 counter receptors, blocked γδ-T cell mediated killing of prostate cancer cells. γδ-T cells lysed prostate cancer cell lines largely through the perforin/granzyme pathway.

Conclusions: Ex vivo, expanded human Vγ9Vδ2+ γδ-T cells are able innately to recognize and kill certain human prostate tumor cell lines in vitro. The recognition and killing of prostate cancer cells occurs in a γδ-T-cell receptor dependent manner and it also appears to involve interactions between ICAM-1 and CD18. Because apoptosis resistant human Vγ9Vδ2+ γδ-T cells can readily be expanded to large numbers (clinical scale), these findings must be considered in the context of developing adoptive immunotherapy strategies to exploit γδ-T cell innate immune responses to prostate cancer.

KEY WORDS: prostate; prostatic neoplasms; T-lymphocytes; cytotoxicity, immunologic; immunotherapy

Although current standard therapies for early stage prostate cancer, including surgery, radiotherapy or hormonal blockade, are usually effective for achieving initial disease control, prostate cancer often recurs. Moreover, salvage chemotherapy for recurrent prostate cancer or chemotherapy for prostate cancer presenting initially as widespread metastatic disease is often associated with poor responses. Clearly new forms of therapy for recurrent or metastatic prostate cancer are needed.

The view that the immune system itself might be exploited for the treatment of prostate cancer is not new. Interestingly to date the overwhelming majority of reports in this regard have focused primarily on augmenting the adaptive immune response to prostate cancer specific antigens. This includes a number of important studies designed to induce tumor specific cytotoxic CD8+ αβ-T lymphocytes (CTLs) using prostate cancer specific peptide antigens as well as other studies designed to develop tumor specific immune responses using dendritic cell based vaccination strategies. However, these and similar approaches that rely primarily on adaptive immunity have several potential shortcomings.

1) These strategies presuppose that an antigen selected as a target for immune based therapy is indeed tumor specific, that is the antigen is expressed only in tumor cells but not in normal tissues. This problem is illustrated when one considers studies designed to target prostate specific antigen (PSA) as a tumor specific immunogen. While PSA is expressed by the majority of prostate cancer cells, it is also expressed by normal prostate tissues. Moreover, PSA might not be expressed at all by some poorly differentiated adenocarcinomas.

2) It is well established that prostate cancer cells can down-regulate the expression of major histocompatibility complex (MHC) class I or II molecules, or have defects in the assembly and expression of MHC class I.1–3 Thus, tumor cells expressing few or no MHC molecules might selectively escape recognition by MHC restricted CD8+ CTL or CD4+ T-helper cells. These latter findings emphasize the importance of developing immunotherapy strategies that do not depend on classic MHC restricted antigen processing and presentation.

Unlike αβ-T cells, γδ-T cells recognize tumor cells in an MHC independent manner, requiring no processing or presentation of tumor specific antigens. Instead, γδ-T cells recognize various MHC class I-like antigens that are commonly shown by cells that have undergone malignant transformation, particularly cells of epithelial origin.4–6 Indeed, γδ-T cells may provide an alternative or complementary means of recognizing and killing tumor cells that have escaped adaptive immune responses.

In peripheral blood γδ-T cells represent only a minor fraction of total T cells, usually only 1% to 5%.7 The majority of peripheral blood γδ-T cells express the Vγ9 T-cell receptor (TCR) chain, usually in combination with the Vδ2 TCR chain (Vγ9Vδ2 γδ-T cells, also termed Vγ2Vδ2 in an alternate no-
mencature). It is thought that Vγ9Vδ2 γδ-T cells provide some degree of immunosurveillance against intracellular pathogens and certain hematological malignancies. In contrast, γδ-T cells found in epithelial tissues, such as the intestine, skin, tongue, esophagus, trachea, lungs and genital tract, usually express the Vδ1 TCR chain in combination with various Vγ chains. Vδ1 γδ-T cells can be found in association with or even infiltrating some solid tumors and they often show MHC unrestricted lytic activity against various cancer cells, particularly those of epithelial origin.

We have previously identified a CD2 mediated, interleukin (IL)-12 dependent signaling pathway that inhibits apoptosis in mitogen stimulated human γδ-T cells derived from peripheral blood. In turn this allows the large-scale expansion of apoptosis resistant γδ-T cells, of which most express Vγ9Vδ2 TCR.

Although it has been reported that activated Vγ9Vδ2 γδ-T cells are capable of in vitro killing of human lymphoma cell lines, little is known regarding the innate antitumor activity of Vγ9Vδ2 γδ-T cells as measured against human epithelial derived cancer cell lines. Herein we describe the innate antitumor activity of ex vivo, expanded human Vγ9Vδ2 γδ-T cells against human prostate cancer cell lines and discuss this finding in the context of developing new strategies for adoptive cellular therapy for prostate cancer.

**Materials and Methods**

*Cell lines.* The human prostate cell lines DU145, LNCaP and PC-3 (American Type Culture Collection, Manassas, Virginia) and the normal human keratinocyte cell line HaCat13 were used.

Preparation of γδ-T and αβ-T cells from peripheral blood mononuclear cell (PBMC) cultures. Ex vivo, expanded, apoptosis resistant γδ-T cells were prepared as previously described. PBMCs were isolated by Ficoll gradient centrifugation of whole blood obtained from healthy human volunteers. Cultures were initiated at a cell density of 1 × 10^6 cells per ml in RPMI-1640 with 10% fetal bovine serum, 2 mM L-glutamine, 100 U/ml penicillin, 100 U/ml streptomycin and 50 μg/ml mercuricethanol. On the day of culture initiation (day 0), 1,000 U/ml human recombinant interferon-γ, 10 U/ml human recombinant IL-12 and 1 to 10 μg/ml mouse antihuman CD2 mAb clone S5.2 (mouse IgG2a) were added. At 24 hours (day 1) cultures were stimulated with 10 ng/ml antiCD3 mAb OKT3 (mouse IgG2a) and 300 U/ml human recombinant IL-2. Fresh medium with 10 U/ml IL-2 was added every 7 days. After 2 weeks γδ-T cells were isolated from cultures by immunomagnetic column using a positive selection strategy. Cells were first stained with antiγδ-TCR mAb conjugated to magnetic beads and then passed through an AutoMACS immunomagnetic cell sorter (Miltenyi Biotec, Auburn, California). αβ-T cells were isolated as the γδ-T cell-depleted fraction. Alternatively αβ-T cells were directly isolated by fluorescence activated cell sorting (FACS) using a high speed cell sorter (FACS DiVa, Becton Dickinson) and directly conjugated antiTCR mAbs. Isolated cells were washed with phosphate buffered saline and cultured overnight in complete RPMI with 100 U/ml IL-2. The purity of isolated γδ-T and αβ-T cells was assessed by FACS and routinely found to be greater than 95% with greater than 90% viability.

**Chromium ⁵¹Cr release cytotoxicity assay.** Target cells were labeled with 100 μCi Na₂ ⁵¹CrO₄ overnight at 37°C, after which cells were washed, trypsinized and suspended in RPMI containing 10% fetal bovine serum. Cells were then plated at 2 × 10³ per well in 96-well V-bottom microtiter trays. Purified αβ-T or γδ-T cells in varying numbers were added to target cells in a final volume of 100 μl. Trays were briefly centrifuged and then incubated for 4 hours at 37°C, after which 50 μl supernatant were removed to determine ⁵¹Cr release in cpm. The percentage of specific target cell lysis was calculated, as described previously. Data are presented as the mean ± SD of triplicate samples. In killing blocking assays 2 μg monoclonal antibodies against Vδ2, Vγ9, γδ-TCR or CD18 as well as their corresponding isotype controls were separately incubated with effector γδ-T cells on ice for 20 minutes prior to interacting with target tumor cells. Anti-intercellular adhesion molecule-1 (ICAM-1) mAb (2 μg) and its isotype control were individually incubated with tumor cells, first on ice for 20 minutes and then mixed with γδ-T cells. When assaying the calcium dependency of γδ-T cell cytotoxicity, 1 mM ethyleneglycoltetraacetic acid (EGTA) and 1.5 mM MgCl₂ were added to cell co-cultures. To restore killing 3 mM CaCl₂ was added in culture with EGTA.

**Flow cytometric analysis of cells.** Flow cytometry analysis was performed as we previously described. Briefly, cells were stained with directly fluoresce conjugated mAbs recognizing TCRVδ1, TCRVδ2 and CD3 separately. Directly conjugated, isotype matched irrelevant antibodies served as controls. Analyses were performed using a FACSCalibur flow cytometer (Becton Dickinson). Propidium iodide uptake was used to exclude nonviable cells. Data analysis was performed using CellQuest software (Becton Dickinson).

**Results**

*Ex vivo, expanded, apoptosis resistant γδ-T cells expressed primarily Vγ9Vδ2 TCR.* The composition of ex vivo, expanded, apoptosis resistant γδ-T cells with respect to TCR Vδ use was determined (fig. 1). A representative study indicated that the majority of ex vivo, expanded, apoptosis resistant γδ-T cells expressed Vδ2 TCR and not Vδ1 TCR (fig. 1, B). As importantly, when compared with methods of T-cell expansion using standard mitogens, the method we used for expanding apoptosis resistant γδ-T cells yielded substantially greater total numbers of γδ-T cells and consequently a greater total number of Vδ2+ γδ-T cells (fig. 1).

Vγ9Vδ2 γδ-T cells showed cytotoxicity activity against some prostate tumor cells. It is established that activated Vγ9Vδ2 γδ-T cells show cytotoxicity activity to lymphoma cell lines. However, the ability of these cells to recognize and lyse malignant cell lines of epithelial origin has not been well described. We examined the cytolytic activity of apoptosis resistant Vγ9Vδ2 γδ-T cells against the human prostate tumor cell lines DU145, PC-3 and LNCaP (fig. 2). PBMCs were isolated from healthy donors and cultured as described. After 14 days apoptosis resistant γδ-T cells were sorted to high purity using an immunomagnetic cell separator. The human prostate cancer cell lines DU145, PC-3 and LNCaP were first labeled with ⁵¹Cr and then incubated with γδ-T cells or control αβ-T cells. After a 4-hour incubation at the indicated effector-to-target (E-to-T) ratios supernatants were removed to determine ⁵¹Cr release in cpm. Data are expressed as the mean percent specific target lysis ± SD of triplicate determinations. These representative findings indicated that apoptosis resistant γδ-T cells can specifically lyse the human prostate cancer cell lines DU145 and PC-3 but not LNCaP.

**Involvement γδTCR in the lysis of sensitive tumor cells by Vγ9Vδ2 γδ-T cells.** Vδ1+ γδ-T cells are able to kill a wide variety of epithelial derived tumor cells in vitro, as has been reported. As described, we observed that Vγ9Vδ2 γδ-T cells are also able to recognize some epithelial tumor cells, such as DU145 and PC-3. To determine whether the γδTCR itself is involved in the recognition and killing of tumor cells blocking antibodies to TCR chain Vγ9 or Vδ2 were added to effector γδ-T cells prior to co-culture with tumor target cells (fig. 3). Each antibody inhibited specific lysis, suggesting that TCR participates in the γδ-T cell mediated lysis of tumor cells. The function of TCR in antitumor activity was also verified in cytotoxicity assays by the addition of pan-γδTCR mAbs, which recognize all γδ-T cells regardless of TCR chain expression.
Involvement of additional cell surface structures in the recognition and lysis of sensitive tumor cells by Vγ9Vδ2 γδ-T cells. Similar studies were performed using a number of mAbs to known surface structures likely to be involved in the interaction between cytotoxic γδ-T cells and the sensitive prostate cancer cell line PC-3. In a number of studies performed only mAbs to CD18 and CD54 were able consistently to inhibit γδ-T cell mediated killing of PC-3 cells, suggesting that interactions between CD18 and CD54/ICAM-1 may be involved in the killing of prostate cancer cells by Vγ9Vδ2 γδ-T cells (fig. 3).

Vγ9Vδ2 γδ-T cell mediated cytolysis of tumor cells involves perforin/granzyme pathway. Granule exocytosis and Fas induced apoptosis are known to be involved in CTL mediated cytotoxicity. Because γδ-T cells constitutively express perforin and granzymes (serine esterases), we examined whether perforin/granzyme exocytosis is a mechanism involved in the lysis of sensitive prostate cancer cells. Since granule exocytosis is Ca2+ dependent, cytolysis assays were performed in the presence of Ca2+ chelators. Figure 4 shows that the lysis of DU145 by Vγ9Vδ2 γδ-T cells was blocked by the addition of EGTA-Mg2+ (depletion of Ca2+) but it was partially restored by Ca2+ replacement. This suggests that the Vγ9Vδ2 γδ-T cell mediated cytolysis of prostate cancer cells involves the perforin/granzyme pathway.

**DISCUSSION**

It has been established that human Vδ1 γδ-T cells can kill sensitive human tumor cells through recognition of the MHC class I related molecules MICA and MICB, which are expressed on malignantly transformed cells. This interaction appears to occur through NKG2D, a receptor expressed by subsets of cytolytic T cells and natural killer cells. MICA and/or MICB also appear to interact directly with Vδ1 TCR.

In this study we report that ex vivo, expanded Vγ9Vδ2 γδ-T cells recognize and kill epithelial derived tumor cells in a γδTCR dependent manner. However, it is still unclear whether MICA and/or MICB are directly involved in this process. Indeed, we observed no difference in MICA and MICB expression on cells sensitive to Vγ9Vδ2 γδ-T cell mediated killing (DU145 and PC-3) and cells that were resistant to killing (LNCaP) (data not shown). However, we observed that CD54/ICAM-1 expression was impaired in the LNCaP cell line, which was not killed by Vγ9Vδ2 γδ-T cells (data not shown). This suggests that ICAM-1 interactions with ligands expressed on Vγ9Vδ2 γδ-T cells may be important in the processes of adhesion to and subsequent killing of sensitive tumor target cells. This is supported by our findings that blocking antibodies to CD18 or CD54/ICAM-1 inhibited γδ-T cell mediated tumor cell lysis. Studies to address this issue directly are underway and will be reported separately.

Our finding that ex vivo, expanded, apoptosis resistant human γδ-T cells can recognize and kill human prostate cancer cells is important for a number of reasons. 1) This finding is consistent with the emerging model that human γδ-T cells can indeed recognize and lyse various human epithelial derived tumors. Herein we report that Vγ9Vδ2 γδ-T cells are quite capable of killing epithelial derived tumor cells, a function that is commonly attributed to Vδ1 γδ-T cells. 2) These findings establish that we can expand large numbers of prostate cancer reactive Vγ9Vδ2 γδ-T cells, a finding that to our knowledge has never been reported until now. Importantly based on initial studies usually only 2 ml PBMCs (1 × 10⁶ cells per ml) derived from 3 to 5 ml fresh blood can be used to generate more than 50 × 10⁶ γδ-T cells, of which the majority are Vγ9Vδ2 γδ-T cells (fig. 1). By extrapolating we calculate that with culture optimization in excess of 1 × 10¹⁰ viable γδ-T cells capable of lysing prostate cancer cells can readily be generated using as starting materials safely obtainable volumes of fresh autologous or allogeneic peripheral blood. These points taken together provide a rationale that which is biologically sound and practical for proposing further studies to determine how human γδ-T cells might be exploited for adoptive immunotherapy for prostate cancer.
These current studies will eventually allow us to address a number of questions that to our knowledge cannot currently be answered. For example, presuming that we can routinely accomplish the clinical scale expansion of tumor reactive αβ-T cells from patients with prostate cancer, will infusing supraphysiological numbers of these αβ-T cells restore or augment innate immune responses against tumors, thus, moderating tumor growth or progression? Can tumor-reactive γδ-T cells be administered alone or will they best be used in conjunction with standard hormone, chemotherapy or radiation based treatments? Only properly designed future clinical trials based largely on such findings reported herein will be able adequately to address these issues.

CONCLUSIONS

Ex vivo, expanded Vγ9Vδ2+ γδ-T cells can innately recognize and kill certain human prostate tumor cell lines in vitro. Prostate cancer cell killing occurs in a γδ-TCR dependent manner and involves interactions between ICAM-1 and CD18. The perforin/granzyme pathway is used by γδ-T cells in the killing of tumor cells. Because apoptosis resistant human Vγ9Vδ2+ γδ-T cells can readily be expanded to large

Fig. 2. Assessment of cytolytic activity of human Vγ9Vδ2 γδ-T cells against human prostate cancer cell lines and control normal human cells. PBMCs were isolated from healthy donors and cultured as described. After 14 days apoptosis resistant γδ-T or control αβ-T cells were sorted high purity using immunomagnetic cell separator. Human prostate cancer cell lines LNCaP, DU145 and PC-3 were labeled with 51Cr and incubated with γδ-T or control αβ-T cells derived from 3 healthy donors, that is 2 male donors (1 and 2) who were 44 and 39 years old, respectively, and female donor (3) who was 38 years old (A). After 4-hour incubation at indicated E-to-T ratios supernatants were removed to determine 51Cr release in cpm. Data are expressed as mean percent specific target lysis ± SD of triplicate determinations. These studies are representative of experiments performed at least 3 other times. Normal human keratinocytes (HaCat cells) were labeled with 51Cr and incubated with γδ-T or control αβ-T cells derived from separate additional healthy donors, that is 2 male donors (a and b) who were 50 and 46 years old, respectively, and female donor (c) who was 25 years old (B). As described, data are expressed as mean percent specific target lysis ± SD of triplicate determinations and are representative of studies performed at least 3 other times. Nonmalignant normal human skin fibroblast cell line CCD-112Sk and normal human hepatic epithelial cell line WRL-68 were similarly not killed by γδ-T or control αβ-T cells (data not shown). Whether isolated by γδ-T cell depletion by immunomagnetic column separation or isolated directly by high speed cell sorting using anti-TCR mAbs control αβ-T cells did not kill tumor or normal control target cells (data not shown). In addition, control αβ-T cells sorted from standard mitogen stimulated cultures or from cultures promoting γδ-T cell expansion did not kill tumor target cells or normal control target cells (data not shown).

Fig. 3. Antibodies to CD18 and CD54/ICAM inhibited Vγ9Vδ2 γδ-T cell mediated tumor lysis. Cytotoxicity assays were performed in presence of anti-TCR-Vδ2, anti-TCR-Vγ9 or anti-TCR-γδ mAbs, or corresponding isotype controls. Effector γδ-T cells were isolated as described and co-cultured with 51Cr labeled cell line DU-145 (A) or PC-3 (B) at E-to-T ratio of 10:1. Similarly effector γδ-T cells were co-cultured with 51Cr labeled PC-3 cells with addition of mAb against CD54/ICAM-1, CD18 or isotype control (C). Relative tumor specific lysis ± SD of triplicate determinations in presence of each specific antibody was calculated by comparison with its corresponding isotype control. Studies are representative of experiments performed at least 3 other times.
numbers (clinical scale), these findings must be considered in the context of developing adoptive immunotherapy strategies to exploit γδ-T cell innate immune responses to prostate cancer.

Dr. Louise Chow, University of Alabama at Birmingham, Birmingham, Alabama provided the HaCat cell line.

REFERENCES

Selected portions from recently awarded Prostate Cancer Foundation grant
Studies from our laboratory have determined that ex vivo expanded human γδ-T cells can innately kill human prostate cancer cells in vitro. Moreover, the introduction of these human γδ-T cells into mice harboring human prostate cancer cells results in the regression of established tumors in these animals. Here we propose studies designed to integrate the biological, clinical and regulatory issues which will be essential for obtaining future FDA approval for first generation clinical trials in which we intend to introduce ex vivo expanded human γδ-T cells as a primary or adjuvant form of treatment for advanced or relapsed prostate cancer.
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BACKGROUND

New Approaches for the Treatment of Recurrent or Advanced Prostate Cancer

Adenocarcinoma of the prostate remains one of the leading causes of cancer-related death among American men. Although current standard therapies for early-stage prostate cancer – including surgery, radiotherapy or hormonal blockade – are usually very effective at achieving initial disease control, prostate cancer often recurs. Moreover, salvage therapy for recurrent prostate cancer – or therapy for prostate cancer presenting initially as wide-spread metastatic disease – is often only modestly effective. Clearly, entirely new forms of therapy for recurrent or metastatic prostate cancer are needed.

One such novel approach is put forth in this application. Here we propose a series of studies designed to allow us to ultimately examine how adoptively transferred human \( \gamma \delta \)-T cells might be exploited as a primary or adjuvant form of therapy for relapsed or advanced stage prostate cancer. The biological rationale for proposing such a cell-based therapeutic approach to prostate cancer is briefly discussed below.

Innate Antitumor Activity of \( \gamma \delta \)-T cells: Biological Basis for New Approaches to Cancer Treatment

Unlike \( \alpha \beta \)-T cells which require the recognition of specific processed peptide antigens presented by major histocompatibility complex (MHC) molecules (adaptive immune response), \( \gamma \delta \)-T cells in contrast, appear to recognize and respond to a variety of stress-induced self antigens commonly displayed by cells which have undergone malignant transformation (1-5). Thus, while incapable of recognizing tumor-specific antigens per se, \( \gamma \delta \)-T cells can nonetheless recognize malignantly transformed cells – particularly malignant cells of epithelial origin – through less specific mechanisms that require no prior antigen exposure or priming (innate immune response). Consequently, human \( \gamma \delta \)-T cells can recognize and lyse malignantly transformed cells immediately upon encounter – consistent with their role as a component of the innate immune system.

Given this, it stands to reason that clinically it might be possible to develop strategies whereby human \( \gamma \delta \)-T cells are administered as a primary or adjuvant form of adoptive cellular immunotherapy for the treatment of various epithelial-derived malignancies, including possibly prostate cancer. Nevertheless, despite the theoretical promise of \( \gamma \delta \)-T cell-based immunotherapy, to date there has been little clinical progress made toward examining how \( \gamma \delta \)-T cells might be directly exploited in such a manner. This has been due in great measure to the lack of biologically rational and clinically practical methods for the isolation or expansion of viable, functional human \( \gamma \delta \)-T cells given their relative infrequency in accessible tissues such as peripheral blood. Moreover, we and others have come to recognize that in vitro, standard culture methods used to expand human T cells are unsuitable for the expansion of \( \gamma \delta \)-T cells as the strong T cell mitogens often employed can directly induce apoptosis in \( \gamma \delta \)-T cells (6-9). That \( \gamma \delta \)-T cells simply die when expanded ex vivo has remained a serious obstacle to the development of \( \gamma \delta \)-T cell–based forms of adoptive cellular immunotherapy.

New Model for the Primary or Adjuvant Therapy of Prostate Cancer?

As reported (9, 10), we initially identified a CD2-initiated signaling pathway which inhibits apoptosis in mitogen-stimulated human \( \gamma \delta \)-T cells. By exploiting this signaling pathway, our laboratory has pioneered the development of the methodologies permitting the reliable and reproducible ex vivo expansion of viable, apoptosis-resistant human \( \gamma \delta \)-T cells. Importantly, these ex vivo expanded \( \gamma \delta \)-T cells retain potent innate antitumor activity against a wide variety of human tumor cell lines in vitro (9, 11, 12), including a number of human prostate cancer cell lines as we have very recently published (13).

Directly relevant to this proposal, we have recently determined that \( \gamma \delta \)-T cells can be reliably expanded from peripheral blood obtained from patients with prostate cancer and that these ex vivo expanded patient-derived \( \gamma \delta \)-T cells retain significant antitumor lytic activity against a variety of human prostate cancer cell lines (manuscript in preparation).

In addition to the above studies, we have since shown that human \( \gamma \delta \)-T cells are able to kill human prostate cancer cells which have first been xenografted into mice (manuscript in preparation). Tumor-bearing mice treated intravenously with human \( \gamma \delta \)-T cells had significant slowing of tumor growth in comparison to control tumor-bearing mice that received no human \( \gamma \delta \)-T cells. In tumor-bearing mice receiving larger or repeated doses of human \( \gamma \delta \)-T cells, complete resolution of tumors was observed in a large proportion of these animals. Importantly, treatment-related adverse side effects were not seen in any animals. Together, these findings highlight the potential importance of developing the means to adoptively transfer human \( \gamma \delta \)-T cells as a form of treatment for prostate cancer.

Pre-clinical and Biological Rationale for Further Studies of \( \gamma \delta \)-T cells in the Context of Prostate Cancer

Recently, it has been observed by ourselves and others that persons diagnosed with certain malignancies (such as melanoma) appear to suffer profound defects within the \( \gamma \delta \)-T cell compartment compared to healthy controls. Directly pertinent to the work proposed in this grant, we have recently generated provocative preliminary data derived from a small series of patients with newly diagnosed, untreated prostate cancer. When compared to healthy individuals, patients with prostate cancer appear to have substantially lower \( \gamma \delta \)-T cell counts (expressed as cells/μl of blood, similar to how one would express a CD4 T cell count in an HIV-infected person). Although these data are still relatively
limited, total γδ-T cell numbers in patients with prostate cancer are substantially lower (mean = 26 cell/µl; n=7) compared to healthy controls (mean = 80 cells/µl; n=8) with a P=0.003 derived using the Student's t-test. Importantly, total lymphocyte counts in patients and controls were not different suggesting that patients have a true loss of γδ-T cells, and not simply a non-specific reduction in the number of circulating lymphocytes.

While it has yet to be established that the loss or impairment of γδ-T cells in humans is permissive for the development of any malignancy, we argue (as stated in our hypothesis, below) that deletion or functional failure of γδ-T cells may contribute to impaired tumor immunosurveillance – a state which is thereupon permissive for either the development or progression of prostate cancer. Significantly, this evolving view is strongly supported by data from our animal models (proof-of-principle studies) in which we have taken a classical genetic approach to demonstrate that γδ-T cells provide important innate antitumor immunosurveillance against prostate cancer: By breeding TRAMP mice (transgenic mice which develop prostate cancer) with mice lacking γδ-T cells (TCRδ−/− mice), we have shown that the absence of γδ-T cells in TRAMP × TCRδ−/− mice is permissive for the more rapid and more aggressive development of prostate cancer in these animals (manuscript in preparation).

Objectives and Goals of this Proposal: Relevance to the Treatment of Prostate Cancer

Ultimately we wish to design and execute clinical studies in order to directly address the following question: Given that we now have the potential to generate large (clinical scale) numbers of prostate cancer-reactive human γδ-T cells, will infusing supra-physiological numbers of these γδ-T cells - either in the autologous or allogeneic setting - augment or restore innate immune responses against prostate cancer?

In this context, the studies proposed in this grant will provide important scientific as well as pre-clinical data necessary for the rational design of our first generation clinical trials. As importantly, these studies will also provide data necessary for future U.S. Food and Drug Administration (FDA) regulatory filings which will be required in order to perform these investigator-initiated clinical trials.

For clarity, the overall objectives of this proposal are stated here in the form of the following hypothesis, a related corollary and a prediction arising from our biological and early pre-clinical models:

**Hypothesis:** By virtue of their ability to innately recognize and kill malignantly transformed cells, γδ-T cells play a role in limiting the development, progression or relapse of prostate cancer (tumor immunosurveillance). As a consequence, a loss in number or function of prostate cancer-reactive γδ-T cells is important in the pathobiology of this disease. Conversely, the administration of supra-physiological numbers of tumor-reactive γδ-T cells may serve to restore or augment innate immune responses against prostate cancer.

**Corollary:** Apoptosis-resistant human γδ-T cells which readily expand in our cultures – yet retain innate, MHC-unrestricted antitumor cytotoxicity – can provide an important means to examine both the pre-clinical biology and the direct clinical potential of human γδ-T cells as a novel form of adoptive cellular immunotherapy for the primary or adjuvant treatment of prostate cancer.

**Prediction:** The large-scale FDA-compliant ex vivo expansion of human γδ-T cells will allow for the clinical administration of cells possessing innate antitumor activity directed against prostate cancer. As such, we will be able to design and execute entirely novel first generation clinical trials for either the primary or adjuvant therapy of prostate cancer. This and related approaches will redefine the strategies used for the treatment of prostate cancer.

**PROPOSED STUDIES**

**Aim 1:** To determine the extent to which a) γδ-T cell numbers; b) γδ-T cell innate antitumor capacity and; c) γδ-T cell expansion potential vary as a function of prostate cancer clinical stage, clinical progression and clinical response to standard therapy. (Clinicopathologic correlations)

**Rationale:** In the setting of certain cancers, such as melanoma, it has recently been noted that both γδ-T cell numbers and γδ-T cell function are substantially diminished (30), a finding which is confirmed by our laboratory. In addition, we have recently observed similar defects in the γδ-T cell compartment in patients with prostate cancer. It is important to note that it has yet to be established whether the loss or impairment of γδ-T cells in humans is permissive for the development of any cancers. However, as stated in our hypothesis, we argue that deletion or functional failure of γδ-T cells may be a critical event leading to impaired antitumor immunosurveillance – a state which would thereupon be permissive for the development or progression of cancers. It is not known if there exists a similar defect in the γδ-T cell compartment in patients with prostate cancer, a critical question to be addressed here.

**Approach:** The studies in this aim are primarily observational (clinicopathologic correlations) and will serve to establish the nature or magnitude of the defects in the γδ-T cell compartment of prostate cancer patients. In subsequent studies (Aim 2), we will extend our model of induced resistance to apoptosis to determine the extent to which tumor-reactive γδ-T cells can be expanded from patients with prostate cancer. Initially, we will accrue study subjects for cross-sectional analysis and thus, will only be able to examine or describe the γδ-T cell compartment in these persons at a static point in time (i.e., initial encounter). To the extent possible (given the limited duration of this award) we will begin to follow selected study subjects longitudinally. Thus, by collecting initial and longitudinal data on a series of prostate cancer...
patients of all stages (as well as healthy controls) we will be able to determine the extent to which variations occur within the γδ-T cell compartment in relation to disease stage, disease progression and in relation to treatments. Data will be obtained at initial encounter and monthly for the duration of the study. All data will be directly correlated with clinical stage at presentation as well as with all relevant clinical history such as maintenance of remission or stable disease, disease progression or disease relapse.

1. Enumeration of fresh, resting γδ-T cells in blood. Peripheral blood samples will be obtained from patients, with a preference for newly diagnosed, untreated individuals. For each patient sample we will derive a total γδ-T cell count expressed in cells/μl of blood (as one would derive a total CD4 helper T-cell count). The functionally distinct γδ-T cell subsets – defined by the expression of either the Vδ1 or Vδ2 chain of the γδ T-cell receptor (TCR) complex – will also be enumerated.

2. Functional studies on fresh blood. In parallel to the above studies, γδ-T cells will be directly isolated from peripheral blood samples utilizing immunomagnetic separation methods. 51Cr-release cytotoxicity assays will be used to assess the ability of fresh, unexpanded γδ-T cells to lyse human prostate cancer cell lines.

Aim 2: To recognize and define key determinants – both biological and practical – which are necessary for the optimal large-scale, FDA-compliant (i.e., clinical) ex vivo expansion and purification of tumor-reactive human γδ-T cells obtained from both healthy individuals as well as from patients with HCC. (Translational studies intended to form the critical bridge to first generation clinical trials)

Rationale: For delivery at a clinical scale, it is likely that large numbers of γδ-T cells will be required – far larger than we have attempted to expand to date. Originally, we determined that peripheral blood mononuclear cells (PBMC) derived from 3 to 5 ml of fresh blood can be used to generate cultures of over 50-100 × 10^9 T-cells containing 40-60% γδ-T cells. By extrapolating, we calculate that with proper culture optimization, in excess of far greater than 1 × 10^9 viable γδ-T cells could readily be obtained after ex vivo expansion using as starting materials, safely obtainable volumes of fresh peripheral blood. This product could then subjected to a variety of positive or negative selection techniques to obtain a final product highly enriched in terms of a defined or selected γδ-T cell subset. In this aim we will address the critical issues of clinical-scale expansion and purification of human γδ-T cells. Upon expansion and purification, γδ-T cells will also be characterized with respect to phenotype and functional capacity. Demonstrating that tumor-reactive γδ-T cells can be efficiently expanded from peripheral blood of patients with prostate cancer – particularly those with advanced or recurrent disease – will provide a strong rationale for undertaking future clinical trials designed to re-infuse ex vivo expanded autologous tumor-reactive γδ-T cells.

Ultimately, as it envisioned that these cells will be used for clinical therapies, it is imperative that all methods and technologies adopted be developed in strict accordance with FDA standards. All studies will be conducted in collaboration with Dr. L. Lamb, co-investigator and director of the newly created UAB Laboratory for Cell and Tissue Engineering (LCTE), a core facility with the capacity to carry out the aseptic, expansion and purification of human cellular materials on a clinical scale in a cGMP-compliant manner.

Approach:

A. Expansion of γδ-T cells. PBMC will be obtained from controls and from patients representing all disease stages. Selected patients will also be studied at various points in their clinical course (longitudinal studies).

1. Culture methods. Large-scale expansion will be performed initially using identical cell densities and identical concentrations of culture components as initially described (9), but simply scaled to larger volumes. As the cost of these scale-up experiments can be prohibitive, once optimal expansion is determined using our standard expansion protocol, we will downwardly titrate each reagent to the lowest effective concentration.

2. FDA-compliant cell expansion systems. The gas-permeable culture bag system manufactured by Lifecell™ (Miltenyi Biotec; Auburn, CA) has been selected on account that it is FDA-compliant and can easily be adapted for our use.

B. Separation and purification of human γδ-T cells. All large-scale cell separation studies will be performed using the CliniMACS immunomagnetic column system (Miltenyi Biotec, Auburn CA) as this device is already approved for the clinical separation of human cells. Antibodies (mAbs) to be used in these studies have been selected partly on the basis that they are or may be obtained in near cGMP-grade at a future date. PBMC will be obtained from patients or from healthy volunteer donors and subjected to large-scale expansion. γδ-T cells will then be purified using the following methods which will ultimately be compared for effectiveness.

1. Methods for positive selection of γδ-T cells (mAb which bind to γδTCR): Specific positive selection methods and reagents include:

   a. Selection of γδ-T cells using anti-γδ mAb directly conjugated to ferromagnetic microparticles: Miltenyi Biotec markets a research grade ferromagnetic microparticle column with particles that are directly conjugated to an anti-TCRγδ mAb. Labeled cells are immobilized on the immunomagnetic column while unlabeled cells pass through. Miltenyi Biotec has no immediate plans to manufacture this mAb for therapeutic use, but has agreed to follow our progress and possibly reconsider at a future date.
2. Methods that deplete γδ-T cells: Immunomagnetic selection with anti-CD4 and anti-CD8 coated microspheres. With an approved investigational new drug application (IND), this combination of antibodies will become available to use for clinical applications using the CliniMACS system. As the vast majority of human γδ-T cells express neither CD4 nor CD8, selecting-out cells that express either CD4 or CD8 essentially enriches for γδ-T cells. Our laboratory has substantial experience with this method of T cell depletion. This approach will likely be the first one employed in our first generation clinical trials.

C. Assessment of expanded and purified apoptosis-resistant γδ-T cells.

1. Composition and purity. Enumeration of total γδ-T cells and γδ-T cell subsets will be performed after expansion and purification. The number of γδ-T cells found in cultures after expansion will be divided by the number of γδ-T cells present at the initiation of cultures. This ratio will be expressed as a "fold expansion" which will allow the quantitative comparison of expansion efficiency of γδ-T cells derived from patients and healthy donors. The percentage of cells in the final expanded/purified product that are γδ-T cells will also be carefully noted.

2. Viability. Expanded and purified γδ-T cells will be assessed for viability (using a variety of standard methods) in order to satisfy FDA requirements of cell viability ≥ 80 percent for cellular product administered to humans.

3. Efficacy and function: Anti-prostate cancer activity of ex vivo expanded γδ-T cells. Apoptosis-resistant γδ-T cells will be assessed from patients will be assessed for their ability to kill human prostate cancer cell lines.

   i. In vitro antitumor activity. Cytolytic activity of ex vivo expanded and purified γδ-T cells will be assessed against a panel of established human prostate cancer cell lines as well as against primary prostate cancer cell lines and fibroblasts lines established from surgical specimens obtained from patients.

   ii. In vivo antitumor activity. Expanded and purified γδ-T cells will be used for in vivo antitumor assays. Human prostate cancer cell lines (including DU-145, PC-3, LNCap) will be used to establish tumors in nude mice. Animals harboring human tumors will then receive ex vivo expanded human γδ-T cells by intravenous (IV) injection and subsequently assessed by a variety of means to determine the extent of tumor regression. An important element of these pre-clinical studies will be the incorporation of bioluminescence imaging techniques to assess in vivo tumor responses in whole living animals. In collaboration with co-investigators Drs. Chaudhuri and Zinn, we have established luciferase-expressing and green fluorescence protein (GFP)-expressing human prostate cancer cell lines.

   Animals treated with human γδ-T cells, as well as control animals, will be assessed by three different means to determine the extent of tumor response. This includes 1) anatomic and histologic inspection; 2) in vivo bioluminescence detection and, 3) survival. Any given experiment will be terminated if tumor-bearing mice are observed to be moribund. To date, we have observed no treatment-related toxicity in mice receiving human γδ-T cells, though all animals will be followed for treatment-related events.

SUMMARY OF MEASURABLE RESULTS

Transition to Clinical Trials: Integration of Biological, Practical and Regulatory Issues

The studies outlined above are specifically intended to integrate the necessary biological, clinical and regulatory issues in order to efficiently secure FDA approval for our first generation investigator-initiated clinical trials. It is important to note that these types of studies are not routinely funded by federal agencies such as the NIH. Thus, the support provided by the Prostate Cancer Foundation will serve as the crucial bridge between our ongoing pre-clinical experimental models (i.e., in vitro and animal-based studies) and the successful design and execution of our first clinical trials to be funded with future grants intended to support such trials.

Time Line and Expected Results

**Months 1-12**  
**Aim 1** studies (clinicopathological correlations) will span the duration of the grant. As noted above, these studies will establish the extent to which functional or numeric deficits occur within the γδ-T cell compartment of patients with prostate cancer and to determine if these deficits correlate with – or are predictive of – progression or relapse of disease.

**Months 1-4**  
**Aim 2A** studies (initial large-scale ex vivo expansion studies) will be completed.

**Months 1-4**  
**Aim 2B** studies (initial large-scale separation and purification studies) will be completed.
MONTHS 4-8
Completed studies of Aim 2A and Aim 2B will be integrated allowing studies of Aim 2C to begin (assessment of expanded and purified apoptosis-resistant γδ-T cells).

MONTHS 6-8
New clinical protocol for the delivery of autologous ex vivo expanded human γδ-T cells in patients with advanced or recurrent prostate cancer will be drafted.

MONTHS 8-12
Investigational New Drug (IND) application describing the ex vivo expansion and purification of human γδ-T cells will be prepared. This IND along with the final version of protocol will be submitted to the FDA.

MONTH 12+
New R21 application will be submitted to the NCI as early as February, 2007. This application will include the FDA-approved IND and clinical protocol, as required. As we ultimately intend to fund our clinical trial using the NCI Exploratory/Development Award (R21) mechanism (PAR-04-155, "Quick-Trial for Novel Cancer Therapies"), funding provided by this Prostate Cancer Foundation award will be key to advancing our pre-clinical work into direct clinical trials.

RELEVANCE OF THESE STUDIES TO PROSTATE CANCER RESEARCH AND TREATMENT

Until now, the relationship between γδ-T cell innate immunity and prostate cancer has remained essentially unexplored. In keeping with the intent of the Prostate Cancer Foundation award, these proposed studies will now extend our evolving biological and pre-clinical models into the area of prostate cancer translational research – with the potential for future direct clinical applications arising from our findings.

No therapeutic interventions are proposed as part of this grant. However, through the efforts of the multidisciplinary collaborative team assembled for these studies – including K. Zinn and T. Chaudhuri (Multi-modality Imaging); L. Lamb (Laboratory for Cell and Tissue Engineering); and D. Urban (Urology) – we anticipate that this work will generate significant new pre-clinical data which will be essential for the design of our first generation clinical trials. In this special context, it is important to note that the Principal Investigator of this grant (R. Lopez, Director of Translational Research, Bone Marrow Transplantation Program, UAB) is trained as an immunologist, an oncologist and a bone marrow transplantation physician. As such, this will clearly facilitate the logical and expeditious translation of this pre-clinical work into direct clinical studies intended to examine how ex vivo expanded human γδ-T cells can be used as either a primary or adjuvant treatment for prostate cancer. Consistent with the goals of the Prostate Cancer Foundation, it is anticipated that such studies will lead to future new treatment options which may directly effect the lives of those suffering from recurrent or advanced stage prostate cancer.