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13. ABSTRACT (Maximum 200 Words)

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. 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 are, *1)* to more precisely 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*. ***Key findings***: *1)* γδ-T cell numbers appear to be diminished in the peripheral blood of patients with prostate cancer; however, it is not yet clear if this is related to the development or progression of disease. 2*)* 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. *3)*. Conversely, we show that adoptively transferred mouse γδ-T cells are capable of moderating the growth of syngeneic mouse prostate cancer cells (cell line TRAMP C2) in vivo. *4)* By creating a red fluorescence protein (RFP)-expressing TRAMP C2 cell line, we have been able to establish in vivo bioluminescence model for the immunotherapy of murine syngeneic prostate cancer. *5)* We demonstrate for the first time the in vivo capacity of human γδ-T cells to kill human prostate cancer cells (PC-3) first xenografted into nude mice.

14. SUBJECT TERMS
Prostate cancer, immunotherapy, cellular therapy, gama/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. 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 are, *1)* to more precisely 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 current period of this grant for which this report is generated (April 1, 2004 to March 31, 2005) 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. Subsequent studies represent the true experimental core of this aim where 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 these patients. Our intent is to 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). We also intend to follow study subjects longitudinally. As such, we will be able to define the dynamic relationships that might exist between *a)* γδ-T cell number, function and expansion capacity and *b)* clinical indices related to progression or relapse of prostate cancer. We may also be able to correlate recovery of γδ-T cells with response to therapy

Findings

- 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 approximately 30 percent 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 however, are based on a small sample size of fewer than 10 patients to date.

We are currently in the process of evaluating the sample size required to draw more definitive conclusions as our null hypothesis is that there is no difference in the mean absolute number of γδ-T cells between patients with prostate cancer and normal healthy donors. Based on published data of healthy individuals and patients with melanoma (who also appear to suffer deficits in γδ-T cell numbers), the mean γδ-T cell count (cell/μl) was 92.9 (standard deviation = 32.6) and 66.1 (standard deviation = 33.8), respectively. Extending from this, we estimate that a sample of 26 individuals per group will allow us to detect a difference between healthy subjects and patients with prostate cancer of at least 26.8 in mean absolute number of γδ T cells with 80% power and 5% significance level based on a two-sided t-test. Studies are currently ongoing in collaboration with Dr. Donald Urban (UAB Urology). We are currently accruing approximately 2 to 3 patients per week (all stages) and thus, expect to be able to formally demonstrate before the end of this funded project if a defect in the γδ-T cell compartment exists in patients with prostate cancer.

On going work related to this task

- Cytolytic activity of ex vivo expanded γδ-T cells derived from patients with prostate cancer. As PBMC samples are obtained from all donors, we routinely expand apoptosis-resistant γδ-T cells using our previously described methods. In order to minimize inter-experimental variability, we have elected to cryopreserve all of our patient-derived expanded γδ-T cells for use in cytotoxicity assays to be performed simultaneously against a fixed panel of target cells at a future date. We have previously determined that cryopreserved γδ-T cells can readily be thawed and that they are not noticeably impaired in their cytolytic capacity.
- Establishment of primary cell lines from patient-derived samples. We have not successfully established primary tumor cell lines from patient-derived surgical specimens. However, this will be become a priority within the next 4 to 6 weeks given our success in establishing an in vivo xenograft model for the killing of PC-3–derived tumors using human γδ-T cells (see ***Figure 5*** below).

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

Initial Objectives and Rationale

Here, we proposed to determine the extent to which various known molecular receptors are used by apoptosis-resistant γδ-T cells to recognize sensitive prostate cancer cells. In addition, we proposed to define the extent to which known adhesion molecules are involved in the cellular interactions between apoptosis-resistant γδ-T cells and sensitive prostate cancer cells. Finally, we proposed to determine the extent to which granule exocytosis or Fas/FasL–mediated mechanisms are utilized by apoptosis-resistant γδ-T cells in the killing of sensitive prostate cancer cells.

Findings

- The key findings related to this task are presented in a recently published manuscript entitled, "Ex vivo expanded human Vγ9Vδ2+ γδ-T cells mediate innate antitumor activity against human prostate cancer cells in vitro". (See **Appendix 1**).

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

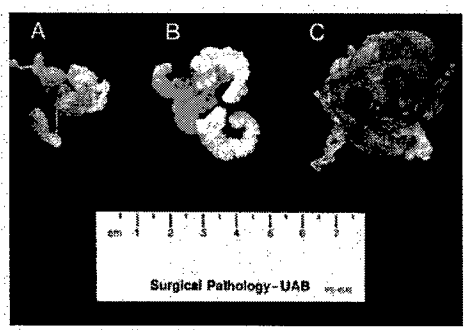
These studies, as outlined in detail in the original proposal, were designed to use mouse models of prostate cancer to define the role of γδ-T cells in controlling the development and growth of prostate cancer. This has been accomplished by classical genetic means utilizing the TRAMP mouse model. In 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: Absence of γδ-T cells is permissive for the development of prostate cancer (proof of principle studies).

- Initial findings (from previous report period): Using the TRAMP transgenic mouse model of prostate cancer, we initially showed that the absence of γδ-T cells is indeed permissive for the development of tumors as demonstrated in ***Figure 1*** (unpublished data). By back-crossing TRAMP mice with mice lacking γδ-T cells (TCRδ$^{-/-}$), we observe that TRAMP × TCRδ$^{-/-}$ mice (***Figure 1C***) develop more aggressive prostate cancers in comparison to control TRAMP animals (***Figure 1B***).
- Follow-up studies: Validation and confirmation of previous preliminary findings (above): While important, the findings from ***Figure 1*** are by themselves, not definitive. Over the last 12 months, we have been able to breed large colonies of both experimental and control mice. Results from ***Figure 2*** (next page) clearly indicate that TRAMP animals which lack γδ-T cells develop larger tumors than TRAMP animals which have a normal γδ-T cell compartment. These statistically significant findings support our view that the absence of γδ-T cells is indeed permissive for the progression of prostate cancer.
- Histological grade of GU tumors from control and experimental mice: All GU tract tissues which were harvested from all animals were fixed in formalin. These samples are in the process of being scored (in a blinded fashion) by our collaborating pathologist with expertise in scoring mouse prostate cancer histology. Over 80 samples are being meticulously scored.

Figure 1. 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. In order to determine if the absence of γδ-T cells is permissive for the development of adenocarcinoma, 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.



Findings, 2: Adoptively transferred mouse γδ-T cells moderate the growth of syngeneic mouse prostate cancer cells in vivo (in vivo models for the adoptive immunotherapy of prostate cancer).

- Establishment of mouse prostate cancer tumors and treatment with syngeneic γδ-T cells. TRAMP C2 cells (hereafter referred to as C2) 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 3*** (page 8) 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 ^{51}Cr-release assays).

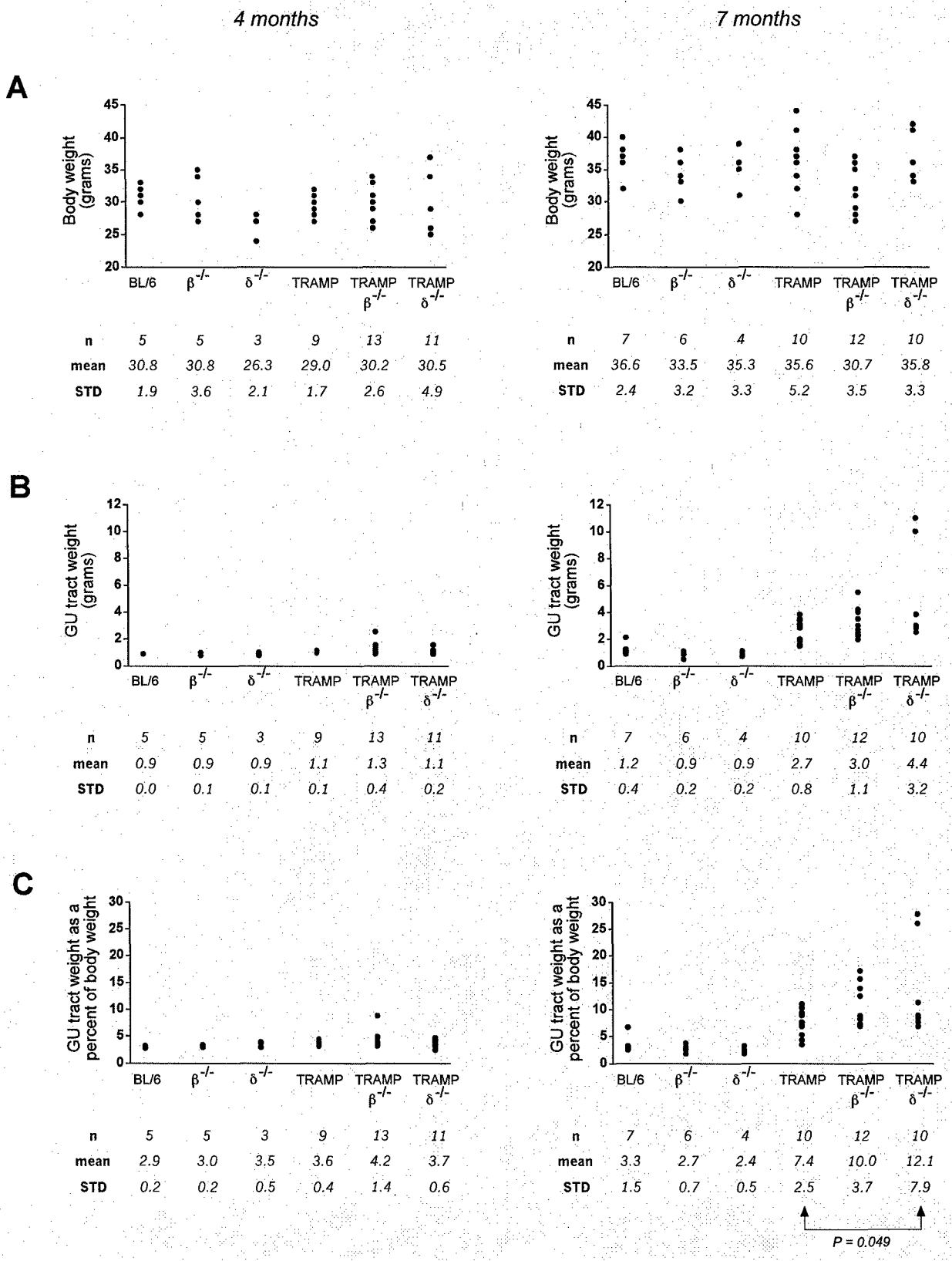


Figure 2 *(legend on following page)*

Figure 2 *(previous page).* **TRAMP animals which lack γδ-T cells develop more extensive genitourinary (GU) tract tumors compared to TRAMP animals with a normal γδ-T cell compartment.**

A) Minimal differences in total body weight between control and experimental animals. Total body weights of individual mice from two cohorts was determined (age 4 months, left; age 7 months, right). All mice are on the C57BL/6 background. Control mice: wild-type mice (indicated as BL/6); TCRβ-deficient mice (indicated as $\beta^{-/-}$); TCRδ-deficient mice (indicated as $\delta^{-/-}$). Experimental mice: TRAMP mice (indicated as TRAMP); TRAMP × TCR$\beta^{-/-}$ mice (indicated as TRAMP$\beta^{-/-}$) and TRAMP × TCR$\delta^{-/-}$ mice (indicated as TRAMP$\delta^{-/-}$). Weights of individual mice in each group are shown graphically. Below each plot, the total number of animals in each group (n) and the mean body weight (± STD) for each group are shown.

B) GU tract weight is greater in TRAMP mice which lack γδ-T cells. GU tract weights of individual mice from the same two cohorts was determined. After sacrifice, GU tracts were removed and weighed. Weights of GU tracts from individual mice in each group are shown graphically. Below each plot, the total number of animals in each group (n) and the mean GU tract weight (± STD) for each group are shown.

C) GU tract weight expressed as a percent of total body weight. For each mouse, the weight of the GU tract was divided by the animal's total body weight in order to express the GU tract weight as a percent of total body weight. GU tract/body weight ratio of individual mice in each group are shown graphically. Below each plot, the total number of animals in each group (n) and the mean GU tract/ body weight ratio (± STD) for each group are shown.

Findings, 2 (continued)

- Establishment of an in vivo bioluminescence model for the immunotherapy of murine syngeneic C2 prostate cancer: ***Figure 4*** (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 and refined in the remaining year of this grant.

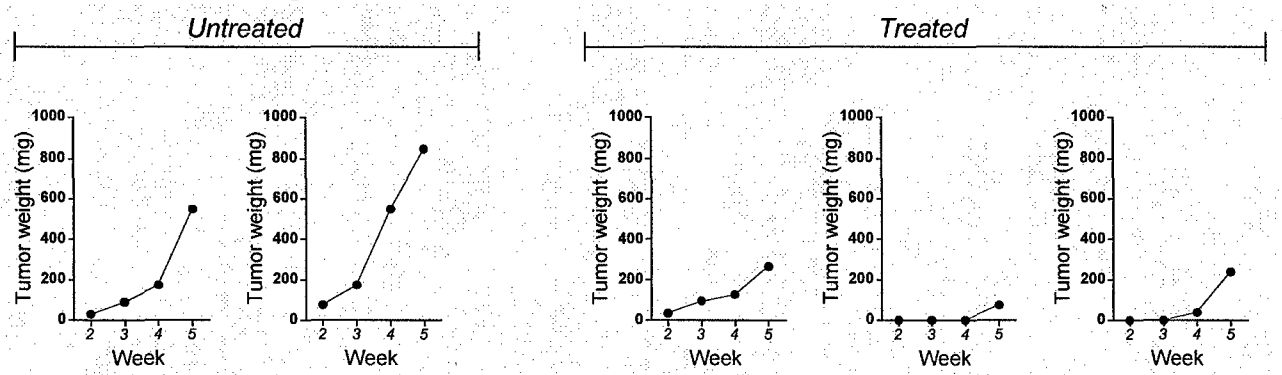


Figure 3. Adoptively transferred mouse γδ-T cells moderate the growth of syngeneic mouse prostate cancer cells in vivo. TRAMP cell line C2 which was derived from TRAMP tumors (a gift of Dr. N. Greenberg) was used to establish tumors in BL/6 mice. Equivalent numbers of tumor cells (1×10^7) were first injected subcutaneously into the thighs of animals. Beginning one week after tumor implantation, individual animals were treated with syngeneic γδ-T cells derived from healthy BL/6 wild-type mice. γδ-T cells were administered intravenously to tumor-bearing animals ("treated", right) on a weekly schedule for a total of four treatments. Cell doses ranged from 0.1 to 1×10^7 cells per treatment. Control animals ("untreated", left) received only PBS injections. Tumor growth was assessed periodically in all animals by calculating tumor weight (mg) based upon two-dimensional measurements using established methods. Data are expressed as tumor weight in mg.

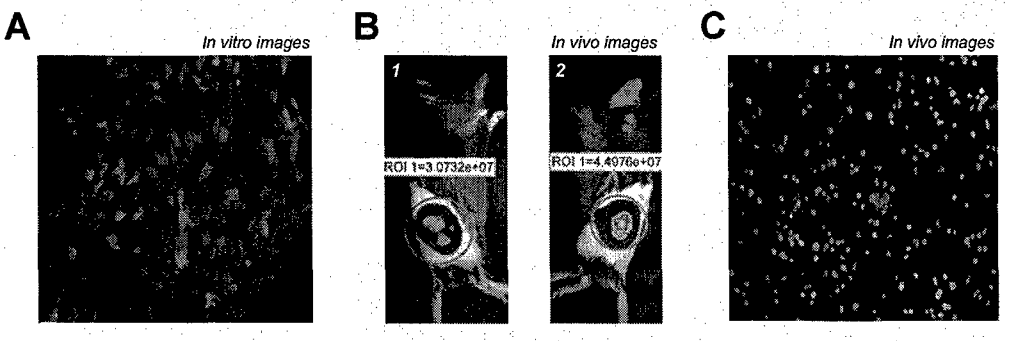


Figure 4. Bioluminescence model for the immunotherapy of murine syngeneic TRAMP C2 prostate cancer: RFP-expressing tumor cells and syngeneic GFP-expressing effector γδ-T cells.

A. Establishment of red fluorescence protein (RFP)-expressing mouse prostate cancer cell line C2.RFP. pCMV-DsRed-Express vector (BD Biosciences) was transfected into TRAMP C2 cells using polyfect transfection reagent kit following manufacture's protocol (Qiagen). Forty eight hours after transfection, cells were split and cultured under G-418 selection. Cells were selected for one month and subsequently subjected to single-cell sorting (FACS DiVa flow cytometer, Becton Dickinson) where RFP-expressing cells were deposited into separate wells of 24 well plates and further cultured under G-418 selection. Clones were subsequently selected and expanded. One representative RFP-expressing TRAMP C2 clone (designated as C2.RFP) is shown here under fluorescence microscopy using the appropriate excitation and emission filters for visualization of RFP.

B. In vivo bioluminescence of wild-type C57BL/6 mice bearing syngeneic C2.RFP-derived tumors. C2.RFP cells (1×10^6) 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 determine the amount of detectable tumor (light emission in photons per second). 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 (ROI) is represented as a pseudo-color scaling of the bioluminescence images. Images of two mice (mouse 1 and mouse 2) are shown. Green circles within each image define the regions of interest (ROI) analyzed.

C. Green fluorescence protein (GFP)-expressing γδ-T cells. Commercially available mice lacking αβ-T cells ($TCR\beta^{-/-}$ mice on the C57BL/6 background, Jackson Labs) were crossed with commercially available mice transgenic for GFP (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, relatively pure populations of GFP-expressing γδ-T cells are readily obtained for further purification or expansion. GFP-expressing γδ-T cells freshly isolated from spleen cell preparations are shown here under fluorescence microscopy using the appropriate excitation and emission filters for visualization of GFP.

Findings, 3: In vivo sensitivity of human prostate cancer cell line PC-3 to human γδ-T cells: Mouse xenograft model (pre-clinical model for the adoptive immunotherapy of human prostate cancer).

- Data in ***Figure 5*** (next page) 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.

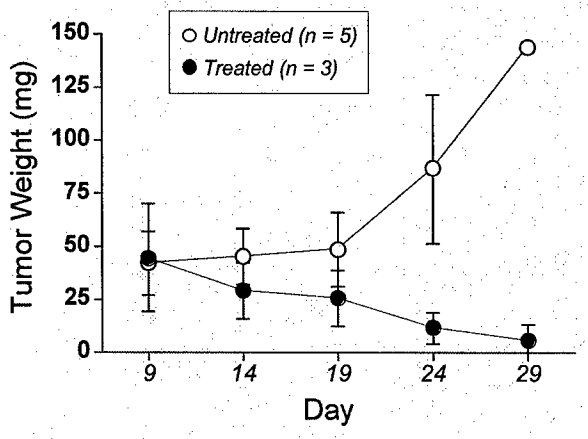


Figure 5. In vivo sensitivity of human prostate cancer cell line PC-3 to human γδ-T cells: Mouse xenograft model. Human prostate cancer cell line PC-3 was used to establish tumors in nude mice. Equivalent numbers of tumor cells (0.5×10^7) were first injected subcutaneously into age-matched and sex-matched 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 . Cell doses 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***).
- Preliminary 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

- Liu Z, Buo BL, Gehrs BC, Nan L and Lopez RD. Ex vivo expanded human Vγ9Vδ2+ γδ-T cells mediate innate antitumor activity against human prostate cancer cells in vitro. *Journal of Urology* 173:1552-1556, May 2005.

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".

REFERENCES

- none

APPENDIX

- Published manuscript attached.

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EX VIVO EXPANDED HUMAN Vγ9Vδ2+ γδ-T CELLS MEDIATE INNATE ANTITUMOR ACTIVITY AGAINST HUMAN PROSTATE CANCER CELLS IN VITRO

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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-

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For another article on a related topic see page 1778.

menclature). It is thought that Vγ9Vδ2 γδ-T cells provide some degree of immunosurveillance against intracellular pathogens and certain hematological malignancies.[8–10] 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.[11] 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.[11]

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.[12] In turn this allows the large-scale expansion of apoptosis resistant γδ-T cells, of which most express Vγ9Vδ2 TCR.

Although it is 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 HaCat[13] 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.[12] 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 mmol/l L-glutamine, 100 U/ml penicillin, 100 U/ml streptomycin and 50 μmol/l mercaptoethanol. 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 ^{51}Cr release cytotoxicity assay. Target cells were labeled with 100 μCi $Na_2\ ^{51}CrO_4$ overnight at 37C, after which cells were washed, trypsinized and suspended in RPMI containing 10% fetal bovine serum. Cells were then plated at 2×10^3 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 37C, after which 50 μl supernatant were removed to determine ^{51}Cr release in cpm. The percent of specific target cell lysis was calculated, as described previouslyl.[12] 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 $MgCl_2$ were added to cell co-cultures. To restore killing 3 mM $CaCl_2$ were added in culture with EGTA.

Flow cytometric analysis of cells. Flow cytometry analysis was performed as we previously described.[12] Briefly, cells were stained with directly fluorescence 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 that 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 ^{51}Cr 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 ^{51}Cr 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.

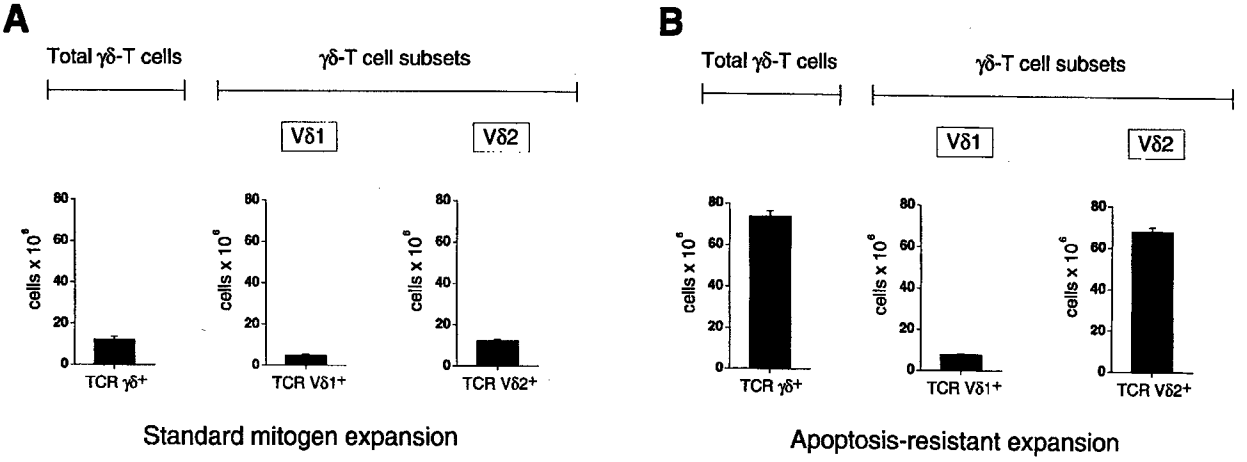

FIG. 1. Composition of ex vivo, expanded, apoptosis resistant gδ-T cells with respect to TCR Vδ use. As we have previously reported,[12] PBMCs were isolated from healthy individuals and then cultured under conditions using standard T-cell mitogens human IL-2 and mitogenic antihuman CD3 antibody OKT3 (*A*) or cultured under conditions promoting expansion of apoptosis resistant γδ-T cells (*B*). Cultures were maintained and expanded as described. After 14 days absolute number of total γδ-T cells was determined in each culture by multiplying total number of cells present by percent of CD3+ cells also staining positive with pan-TCRγδ mAb. In parallel absolute number of Vδ1+ and Vδ2+ γδ-T cells was determined by multiplying total number of cells per culture by percent of CD3+ cells staining positive with mAbs specific for TCR Vδ1 and TCR Vδ2, respectively. Determinations were performed in triplicate on samples. Data are presented as mean absolute number ± SD of total γδ-T cells, and indicate Vδ1+ and Vδ2+ γδ-T cell subsets. Result are representative of experiments performed at least 3 separate times using PBMCs derived from healthy donors. In addition, ex vivo, expanded, apoptosis resistant γδ-T cells were subsequently isolated from cultures by immunomagnetic column separation. After separation cells were maintained in culture for 24 hours prior to analysis to allow mAb used for sorting to dissociate. Re-analysis using fluorescein isothiocyanate conjugated antiVδ1, phycoerythrin conjugated antiVδ2 mAb and Allophycocyanin (APC) conjugated antiCD3 mAb confirmed that expanded, apoptosis resistant γδ-T cells were primarily Vδ2+ (data not shown). As previously reported,[12] viability of freshly purified, expanded, apoptosis resistant γδ-T cells was routinely 90% or higher.

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 esterase), we examined whether perforin/granzyme exocytosis is a mechanism involved in the lysis of sensitive prostate cancer cells. Since granule exocytosis is Ca^{2+} dependent, cytolysis assays were performed in the presence of Ca^{2+} chelators. Figure 4 shows that the lysis of DU145 by Vγ9Vδ2 γδ-T cells was blocked by the addition of EGTA-Mg^{2+} (depletion of Ca^{2+}) but it was partially restored by Ca^{2+} replacement. This suggests that the Vγ9-Vδ2 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.[14] 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.[4–6,15]

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[12] usually only 2 ml PBMCs (1×10^6 cells per ml) derived from 3 to 5 ml fresh blood can be used to generate more than 50×10^6 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 1×10^9 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.

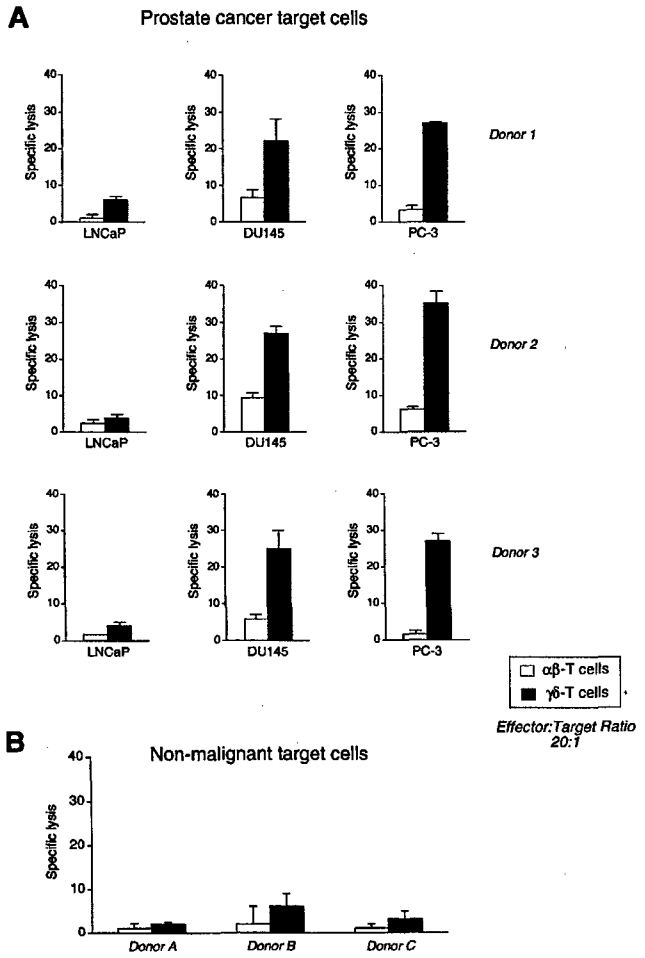

FIG. 2. Assessment of cytolytic activity of human Vγ9Vδ2 γδ-T cells against human prostate cancer cell lines and control normal human cells. PBMCs were isolated from healthy donors and cultured as described. After 14 days apoptosis resistant γδ-T or control αβ-T cells were sorted to high purity using immunomagnetic cell separator. Human prostate cancer cell lines LNCaP, DU145 and PC-3 were labeled with ^{51}Cr 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 ^{51}Cr 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 ^{51}Cr 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-1128sk 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 antiTCR 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).

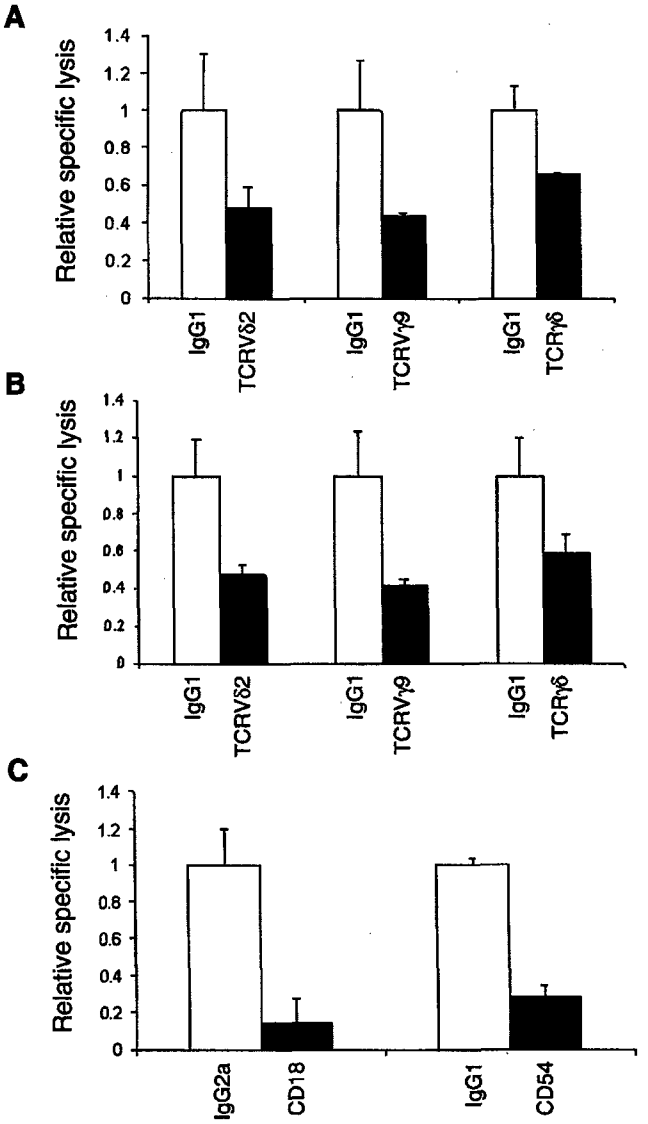

FIG. 3. Antibodies to CD18 and CD54/ICAM inhibited Vγ9Vδ2 γδ-T cell mediated tumor lysis. Cytotoxicity assays were performed in presence of antiTCR-Vδ2, antiTCR-Vγ9 or antiTCR-γδ mAbs, or corresponding isotype controls. Effector γδ-T cells were isolated as described and co-cultured with ^{51}Cr labeled 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 ^{51}Cr 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.

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 γδ-T cells from patients with prostate cancer, will infusing supraphysiological numbers of these γδ-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

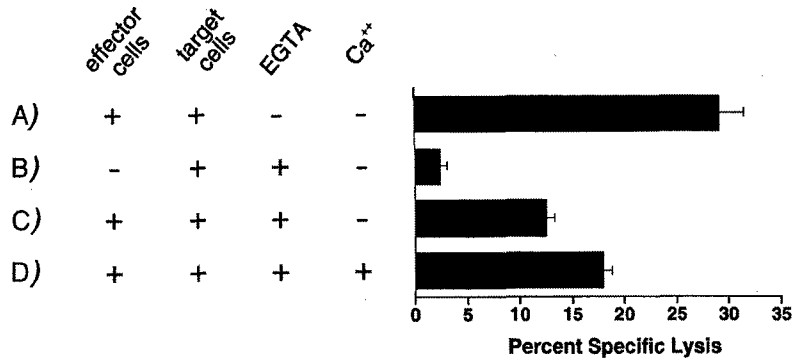

FIG. 4. Cytolysis of prostate cancer cells by Vγ9Vδ2 γδ-T cells involves perforin/granzyme pathway. Standard 4-hour cytotoxicity assays were performed using ex vivo, expanded Vγ9Vδ2 γδ-T cells as effector cells and ^{51}Cr labeled DU145 cells as target cells at 10:1 E-to-T ratio. *A*, effector γδ-T cells co-cultured with ^{51}Cr labeled DU145 cells in RPMI. *B*, ^{51}Cr labeled DU145 cells cultured without γδ-T cells. *C*, effector γδ-T cells co-cultured with ^{51}Cr labeled DU145 cells in absence of Ca^{2+}, depleted by addition of EGTA. *D*, effector γδ-T cells co-cultured with ^{51}Cr labeled DU145 cells with Ca^{2+} replaced.

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.

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