ANTICANCER RESEARCH *40*: 5481-5487 (2020)
doi:10.21873/anticanres.14559

Sphere-derived Prostate Cancer Stem Cells Are Resistant to γδ T Cell Cytotoxicity

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Abstract. *Background/Aim: γδ T cells mediate cytotoxicity against prostate cancer (PCa) cells in vitro; however, the clinical efficacy of γδ T cell-targeted immunotherapy for recurrent and metastatic PCa is unsatisfactory. We hypothesized that the resistance of recurrent and metastatic PCa to γδ T cells is related to the presence of prostate cancer stem cells (PCSCs), and we examined their relationship. Materials and Methods: PCa spheres (prostaspheres) were generated from five PCa cell lines, and their susceptibility to cytotoxicity by γδ T cells was investigated. Expression of stemness-related markers was evaluated by qRT-PCR. Results: Prostasphere-derived cancer cells were resistant to lysis by γδ T cells and expressed higher levels of several stemness markers, including CD133, NANOG, SOX2, and OCT4, than the parental PCa cell lines. Conclusion: Ex vivo-expanded γδ T cells are not effective against PCSCs.*

Prostate cancer (PCa) is one of the most common cancers in men worldwide (1). Although patients with localized PCa are expected to be cured by surgery, radiotherapy, or androgen deprivation therapy (ADT), it is difficult to achieve complete tumor regression in patients with recurrent or metastatic PCa. ADT, the main treatment for these patients, often leads to initial tumor regression, but most patients eventually become resistant and the tumor recurs (2, 3). New chemotherapy regimens and second-generation ADT have been developed over the past decade, but even responder patients eventually become resistant to these therapies (4-6). Therefore, new therapeutic strategies for advanced PCa are needed.

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Key Words: γδ T cell, immunotherapy, prostate cancer, cancer stem cell, sphere, prostasphere, CD133, NANOG, SOX2, OCT4.

Human γδ T cells constitute only a small percentage of circulating lymphocytes, but several methods for their expansion and stimulation have been developed. Although human γδ T cells mediate cytotoxicity against various tumor types, including PCa, *in vitro* and in mouse models *in vivo* (7-11), the clinical efficacy of γδ T cell immunotherapy for metastatic PCa is unsatisfactory (12). Recent studies have indicated that cancer stem cells (CSCs) play an important role in tumor resistance to conventional ADT, radiotherapy, and chemotherapy in various solid tumors, including PCa (13-15). CSCs are a rare cell population within the tumor, but are spared even after conventional therapy because of their resistance and their capacity to self-renew, ultimately causing tumor relapse and metastasis. We hypothesized that the resistance of recurrent and metastatic PCa to γδ T cells was associated with the presence of CSCs. The cytotoxic activity of human γδ T cells against prostate CSCs (PCSCs) has not been reported. The present study aimed to investigate the relationship between resistance of recurrent and metastatic PCa to γδ T cells and PCSCs.

Materials and Methods

Cell lines and culture. The androgen receptor (AR)-positive PCa cell lines LNCaP, C4-2, C4-2B, and 22Rv1, and the AR-negative PCa cell lines DU145 and PC3, were purchased from American Type Culture Collection (Manassas, VA, USA). All cancer cell lines were cultured in RPMI1640 (FUJIFILM Wako Pure Chemical Corporation, Osaka, Japan) supplemented with 10% heat-inactivated fetal bovine serum (FBS; Sigma-Aldrich, St. Louis, MO, USA) and 1% penicillin and streptomycin (PC/SM; FUJIFILM Wako Pure Chemical Corporation). Cells were kept at 37˚C in a 5% CO_2 atmosphere

Ex vivo expansion of human γδ T cells. All healthy donors provided written, informed consent in accordance with Declaration of Helsinki for the use of their peripheral blood for research purposes, and the study was approved by the Kyoto Pharmaceutical University Review Board. Human γδ T cells were expanded as previously described (11). Briefly, peripheral blood mononuclear cells (PBMCs) were isolated from whole blood using Ficoll-Paque (Cytvia/GE Healthcare, Marlborough, MA, USA). PBMCs were

stimulated with 5 μM zoledronic acid (ZOL; Novartis, Basel, Switzerland) and cultured in AlyS505N medium (Funakoshi, Tokyo, Japan) supplemented with 10% heat-inactivated blood group AB human serum for 11 days. Recombinant human interleukin-2 (rhIL-2; 100 U/ml; FUJIFILM Wako Pure Chemical Corporation) was added every day during the culture period, and the amount of culture media was appropriately scaled up as the cells expanded. Cells were analyzed by flow cytometry on day 11, and CD3+/TCR γδ+ cells were considered γδ T cells.

Flow cytometric analysis. Expanded γδ T cells (1.0×10^6 cells in 100 μl PBS) were stained with the following fluorochrome-conjugated monoclonal antibodies (mAbs): FITC-conjugated anti-CD3 (clone UCHT1; BD Biosciences, Franklin Lakes, NJ, USA), PE-conjugated anti-T cell receptor (TCR) αβ (clone IP26, BD Biosciences), and APC-conjugated anti-TCR γδ (clone B1, BD Biosciences). After 30 min on ice in the dark, cells were washed twice and the pellet was resuspended in 500 μl PBS and examined by flow cytometry on an LSR Fortessa (BD Biosciences). Data were analyzed using Flowjo software (BD Biosciences).

Prostate cancer sphere (prostasphere) culture. LNCaP, C4-2, C4-2B, 22Rv1, DU145, and PC3 cells were seeded in non-coated 90 mm dishes (Ina Optika, Osaka, Japan) at a density of 1.0×10^5 cells/ml in 10 ml serum-free DMEM/F12 (FUJIFILM Wako Pure Chemical Corporation) supplemented with B-27 (Thermo Fisher Scientific, Waltham, MA, USA), 20 ng/ml basic fibroblast growth factor (b-FGF; PeproTech, Rocky Hill, NJ, USA), 20 ng/ml epidermal growth factor (EGF; PeproTech), and 1% PC/SM. The culture media was fully replaced on day 4 and scaled up on day 7, and prostaspheres were harvested on day 10.

Quantitative real-time polymerase chain reaction (qRT-PCR). Total RNA was extracted from PCa cells grown in monolayers and from PCa prostaspheres with the NucleoSpin RNA kit (Macherey-Nagel, Dürer, Germany) according to the manufacturer's protocol. RNA was quantified on a NanoDrop 2000 (Thermo Fisher Scientific). Complementary DNA (cDNA) was synthesized using the Revertra Ace qPCR RT kit (Toyobo, Osaka, Japan), and qRT-PCR was performed on the Thermal Cycler Dice Real Time SystemII(Takara, Shiga, Japan). PCR amplification was performed as follows: denaturation at 95˚C for 5 s, and annealing and extension at 60˚C for 30 s. PCR was terminated after 40 cycles. The primer sequences and probes were designed using the Universal Probe Library (UPL). The primers used in this study were as follows:
CD133, forward: 5'-GGAAACTAAGAAGTATGGGAGAACA-3'; reverse: 5'-CGATGCCACTTTCTCACTGAT-3'. NANOG, forward: 5'-ATGCCTCACACGGAGACTGT-3'; reverse 5'-CAGGGCTG TCCTGAATAAGC-3'. SOX2, forward: 5'-GGGGGAATGGAC CTTGTATAG-3'; reverse 5'-GCAAAGCTCCTACCGTACCA-3'. OCT4, forward: 5'-GAAACCCACACTGCAGATCA-3'; reverse 5'-CGGTTACAGAACCACACTCG-3'. 18S rRNA, forward: 5'-GCAATTATTCCCCATGAACG-3'; reverse 5'-GGGACTTAATCA ACGCAAGC-3'. The relative expression of CSC markers in each sample was normalized to that of 18S rRNA (internal control).

Cytotoxicity assays. Cytotoxicity was measured by flow cytometry using carboxyfluorescein succinimidyl ester (CFSE; Dojindo Corporation, Kumamoto, Japan) and propidium iodide (PI; FUJIFILM Wako Pure Chemical Corporation). Target prostaspheres were dissociated into single cells using Accutase (Innovative Cell Technologies, San Diego, CA, USA). Then, the prostasphere-derived single cells and parental PCa cells were labeled with 0.5 μM CFSE for 30 min. CFSE-labeled cancer cells were centrifuged, washed, resuspended in culture medium, and seeded in 24-well plates at 1.0×10^5 cells per well in 500 μl medium containing 5 μM ZOL. After 24 h, *ex vivo*-expanded γδT cells were added to each well. Following 4 h of co-culture, samples were collected, washed, and resuspended in 500 μl PBS containing 5 μl PI (1 mg/ml) for flow cytometric analysis. Samples were read on an LSR Fortessa and the PI+/CFSE+ cells were counted as apoptotic target cells.

Statistical analysis. Statistical analyses were performed using GraphPad Prism 5.0 (GraphPad Software, San Diego, CA, USA). Continuous variables were analyzed using Student's *t*-tests. Multiple comparisons between groups were performed using Bonferroni/Dunn's tests. *p*-Values <0.05 were considered statistically significant.

Results

Ex vivo culture of human PBMCs with ZOL and rhIL-2 expanded human γδ T cells. ZOL and rhIL-2 are known to induce the expansion of human γδ T cells (10, 11). Human PBMCs were cultured in AlyS505N medium supplemented with 10% human AB serum and 5 μM ZOL. Cells were serially re-stimulated by the addition of rhIL-2 (100 U/ml) every day and scaled up by increasing the volume of the medium. On day 11, mature, expanded γδ T cells were harvested and viably frozen. Flow cytometric analysis showed that on day 11, the cell cultures were highly enriched in $CD3^+$/TCR $\gamma\delta^+$ cells (γδ T cells) (Figure 1A). Moreover, the absolute cell number of γδ T cells increased approximately 2000-fold (Figure 1B). Therefore, this *ex vivo* expansion protocol using ZOL and rhIL-2 might yield sufficient numbers of human γδ T cells for adoptive immunotherapy.

Human γδ T cells exhibited cytotoxicity against various PCa cell lines. The cytotoxicity of human γδ T cells against six PCa cell lines (LNCaP, C4-2, C4-2B, PC3, DU145, and 22Rv1) was evaluated by flow cytometry using CFSE/PI double staining. First, PCa cells were labeled with CFSE, pretreated with ZOL, and seeded in culture plates. On the following day, γδ T cells were added at the indicated effector/target (E/T) ratios. After 4 h of co-culture, PI uptake was used to distinguish living cells from dead cells. In all of the PCa cell lines, ZOL treatment increased the percentage of PI+ cells after co-culture with γδ T cells (Figure 2). Thus, the expanded human γδ T cells exerted potent cytotoxicity against PCa cells.

Prostasphere-derived cancer cells were resistant to lysis by human γδ T cells. We were able to generate prostaspheres from five of the six PCa cell lines (LNCaP, C4-2, C4-2B, DU145, and 22Rv1) by culturing them in suspension in serum-free medium supplemented with b-FGF, EGF, and B-27 for 10 days (Figure 3A). Prostaspheres could not be

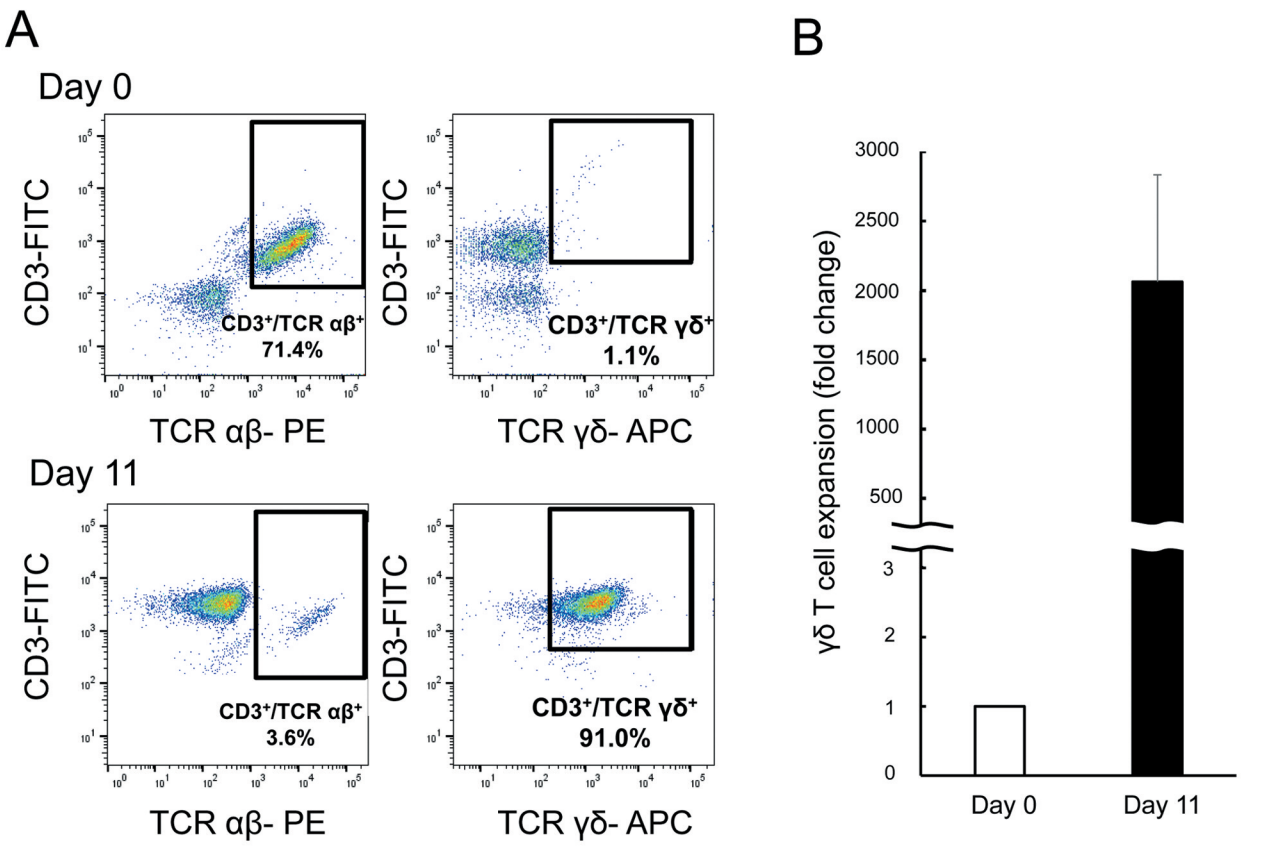

Figure 1. *Ex vivo expansion of human γδ T cells from PBMCs. (A) Representative flow cytometry data of isolated PBMCs from one healthy volunteer, gated on lymphocytes by forward scatter (FSC) and side scatter (SSC). A major subset of CD3+/TCR αβ+ cells (αβ T cells) and a minor subset of CD3+/TCR γδ+ cells (γδ T cells) were observed on day 0 (upper panels). Expansion for 11 days resulted in highly enriched CD3+/TCR γδ+ populations (lower panels). (B) The increase in γδ T cells is expressed as the fold change, relative to the absolute cell number on day 0 (a white bar). Expansion for 11 days resulted in an approximately 2000-fold increase in the number of γδ T cells (a black bar).*

generated from PC3 cells using this culture method because they adhere to the non-coating dishes. To investigate the cytotoxicity of human γδ T cells against prostasphere-derived cancer cells, prostaspheres were digested into single cells, and the cytotoxicity of γδ T cells against the cancer cells was analyzed by flow cytometry, as described above for PCa cells grown in monolayers. The percentages of PI+ prostasphere-derived cancer cells were significantly less than those of the respective parental PCa cell lines (Figure 3B). These results demonstrate that human γδ T cell-resistant PCa cells were generated under sphere-forming conditions.

Expression of stemness-related markers were increased in γδ T cell-resistant PCa cells. The expression of stemness-related markers in γδ T cell-resistant PCa prostaspheres and PCa cell lines was evaluated by qRT-PCR. Several stemness markers, including CD133, NANOG, SOX2, and OCT4, were expressed at higher levels in the γδ T cell-resistant PCa prostaspheres than in the γδ T cell-sensitive parental PCa cells in the C4-2, DU145, and 22Rv1 cell lines (Figure 4) as well as in the LNCaP and C4-2B cell lines (data not shown).

Discussion

The main objective of this study was to generate γδ T cell-resistant PCa cells and examine the expression of stemness-related markers to investigate the relationship between resistance of recurrent and metastatic PCa to γδ T cells and PCSCs. For this purpose, we first expanded human γδ T cells from human PBMCs *ex vivo* and confirmed their ability to kill PCa cell lines. ZOL inhibits farnesyl pyrophosphate synthase (FPPS) in peripheral blood monocytes, resulting in the accumulation of isopentenyl pyrophosphate (IPP), which stimulates the activation and expansion of γδ T cells (16, 17). ZOL also causes IPP accumulation in cancer cells. Human γδ T cells recognize this accumulated IPP through the Vγ9Vδ2 T cell receptor and kill the cancer cells (18, 19). In the present study, our culture method using ZOL and rhIL-2 induced

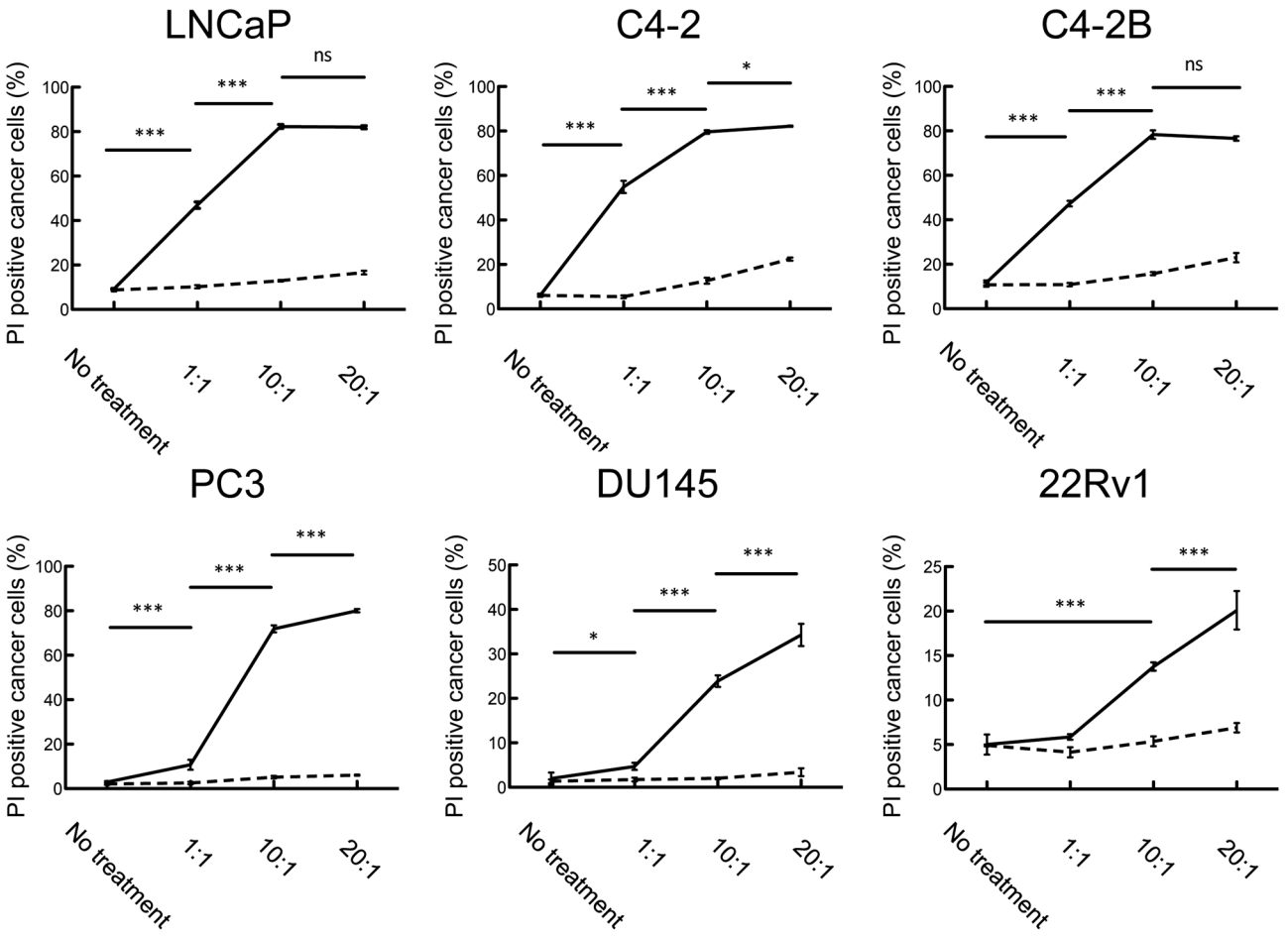

Figure 2. *Human γδT cells exhibit cytotoxicity against six PCa cell lines pretreated with ZOL in vitro. The results are expressed as the percentage of PI+ cancer cells (mean±SD; n=3) after 4 h of co-culture with human γδT cells at various effector:target (E/T) ratios. Dotted lines represent cytotoxicity against cancer cells without ZOL treatment (–). Solid lines represent cytotoxicity against ZOL-treated cancer cell lines (+). *p<0.05; **p<0.01; ***p<0.001; ns, not significant.*

significant expansion of γδ T cells from human PBMCs. Furthermore, these γδ T cells were cytotoxic against all six PCa cell lines when the PCa cells were pretreated with 5 μM ZOL. Several studies have reported the cytotoxicity of γδ T cells against PCa cells *in vitro*. Nicol and colleagues reported that *ex vivo*-expanded γδ T cells had little cytotoxicity against DU145 cells (20). However, Mattarollo and colleagues reported that *ex vivo*-expanded γδ T cells could exert cytotoxicity against DU145 cells, and the cytotoxicity reported in that study was higher than that observed here (9). In the Nicol study, the cancer cells were not pretreated with ZOL, while in the Mattarollo study, the cancer cells were pretreated with a 10-fold higher concentration of ZOL (50 μM) compared to our study (5 μM). These studies suggest that pretreatment of tumor cells with ZOL is important for γδ T cell cytotoxicity, and that the amount of cytotoxicity is related to the ZOL concentration. We chose to pretreat cancer cells with 5 μM ZOL because ZOL was not toxic to PCa cells at this concentration (Figure 2), which enabled us to specifically evaluate the cytotoxicity of the γδ T cells. Our data confirm that *ex vivo*-expanded γδ T cells can exert cytotoxicity against PCa cells.

We next generated prostaspheres and evaluated the cytotoxicity of *ex vivo*-expanded γδ T cells against prostasphere-derived PCa cells. Sphere-forming cultures expose cancer cells to suboptimal conditions, including hypoxia and low nutrient levels, and influence cell-cell contact. These suboptimal conditions capture the biological characteristics of tumors better than conventional adherent culture conditions, and sphere-derived cell cultures show clinically relevant chemoresistance and radioresistance (21, 22). In the present study, prostasphere-derived PCa cells were resistant to *ex vivo*-expanded γδ T cells, even though these cells exhibited cytotoxic activity against the parental PCa cell lines.

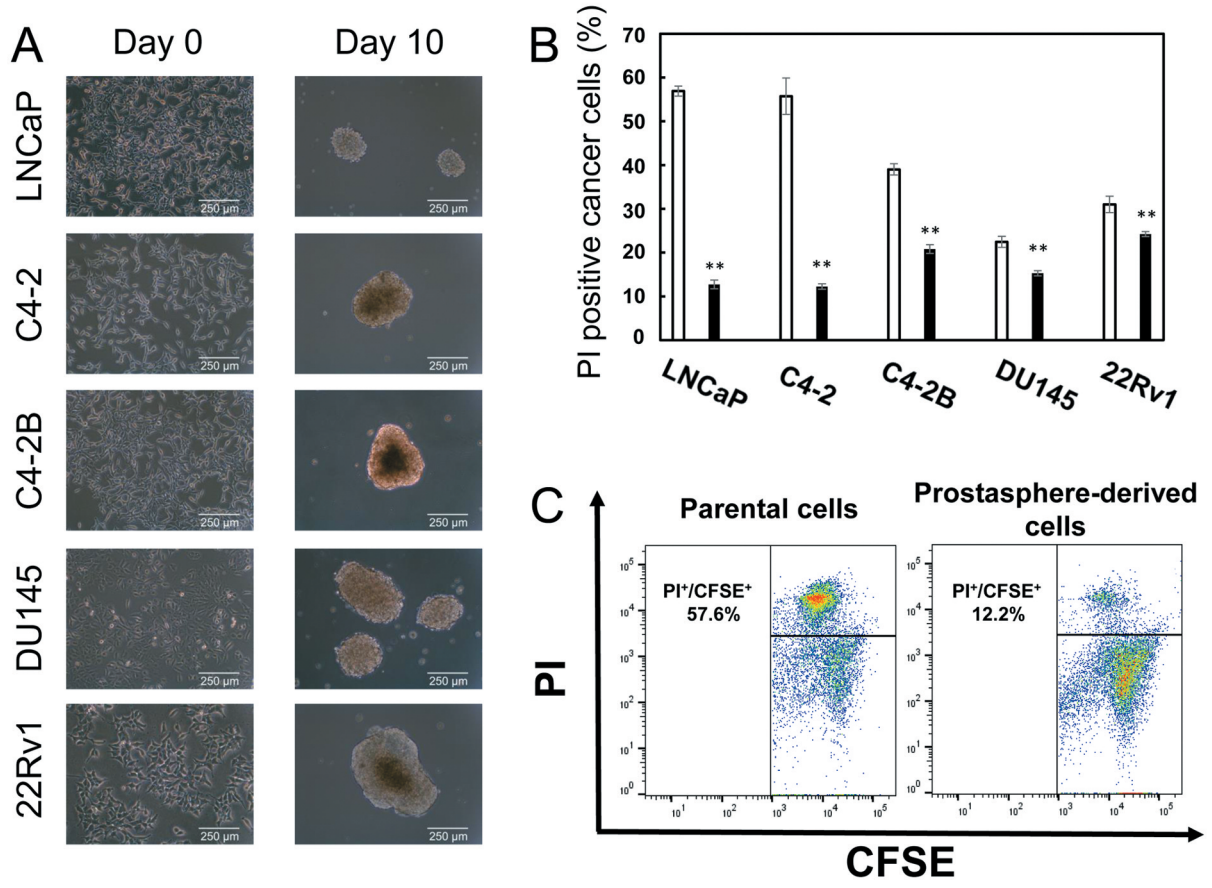

Figure 3. *Prostasphere-derived cancer cells are resistant to lysis by human γδT cells. (A) Microscopic images of parental PCa cell monolayers (day 0, left panels) and prostaspheres (day 10, right panels). (B) Results are expressed as the percentage of PI+ cancer cells (means±SD; n=3) after 4 h of co-culture with human γδ T cells. All PCSCs were pretreated with 5 μM ZOL prior to use in cytotoxicity assays. Comparison of the cytotoxicity against parental PCa cells grown under traditional adherent culture conditions (white bars) and prostasphere-derived cancer cells (black bars). **p<0.01. (C) Representative flow cytometry data from LNCaP cells, as summarized in (B). PI+ cells among CFSE+ tumor cells were counted as apoptotic target cells.*

Finally, we confirmed that the expression of several CSC markers, including CD133, NANOG, SOX2, and OCT4, was increased on prostaspheres compared to parental PCa cells. Expression of these markers by PCa cells is related to CSC characteristics and resistance to ADT and chemotherapy (23-28). ADT and stem cell-related signaling inhibitors have been reported to have synergistic anti-tumor effects against ADT-resistant PCa *in vitro* and *in vivo* (29, 30). It may therefore be possible to achieve synergistic anti-tumor effects by combining γδ T cell-directed immunotherapy with inhibitors of stem cell-related signaling.

Taken together, we showed that prostasphere-derived PCa cells had increased expression of stemness-related markers and were resistant to lysis by human γδ T cells. These results suggest that *ex vivo*-expanded γδ T cells will not be effective against PCSCs. Further research is needed to clarify the mechanisms of PCSCs resistance to human γδ T cells. Future studies in our laboratory will investigate the activation of stem cell signaling in PCSCs for developing combination therapies targeting these mechanisms.

Conflicts of Interest

The Authors have no conflicts of interest to disclose regarding this study.

Authors' Contributions

Conception and design: Masatsugu Miyashita, Osamu Ukimura, and Eishi Ashihara; Experiments and Data analysis: Masatsugu Miyashita, Mako Tomogane, Yuichi Nakamura, and Teruki Shimizu; Interpretation: Masatsugu Miyashita, Atsuko Fujihara, and Eishi Ashihara; Article Writing: Masatsugu Miyashita, Osamu Ukimura, and Eishi Ashihara; Final approval of article: All Authors.

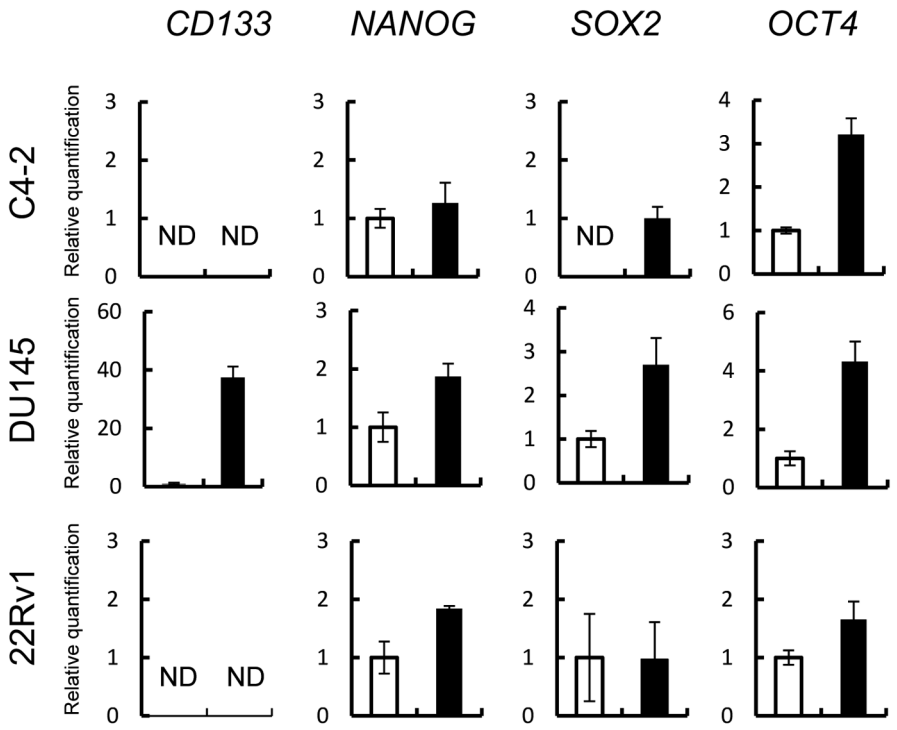

Figure 4. *Human γδT cell-resistant PCa cells showed enhanced mRNA expression of stemness-related markers. Expression of stemness-related markers in parental PCa cells (white bars) and γδ T cell-resistant prostaspheres (black bars) in the C4-2, DU145, and 22Rv1 cell lines by qRT-PCR (means±SE; n=3). ND, not detected. The expression of 18S rRNA was used as the internal control and the ΔΔCT method was used for relative quantification.*

Acknowledgements

The Authors thank Shigekuni Hosogi, MD, PhD; Yuki Toda, PhD; Yusuke Sano; Daiki Shimizu; Ryosuke Wakabayashi; Keigo Amari; and Natsuki Imayoshi (Department of Clinical and Translational Physiology, Kyoto Pharmaceutical University), as well as Saya Ito, PhD, and Yukako Morioka (Department of Urology, Kyoto Prefectural University of Medicine) for technical advice and assistance. The authors also thank Susumu Nakata, MD, PhD, (Department of Clinical Oncology, Kyoto Pharmaceutical University) for providing the protocol for sphere-forming cultures.

Funding

This work was partly supported by the MEXT-Supported Program for the Strategic Research Foundation at Private Universities, 2015-2019 (S1511024L to E.A.), and JSPS KAKENHI (26461436 and 19K08826 to E.A., 20K18123 to T.S).

References

1 Bray F, Ferlay J, Soerjomataram I, Siegel RL, Torre LA and Jemal A: Global cancer statistics 2018: GLOBOCAN estimates of incidence and mortality worldwide for 36 cancers in 185 countries. CA Cancer J Clin *68(6)*: 394-424, 2018. PMID: 30207593. DOI: 10.3322/caac.21492

2 Lytton B: Prostate cancer: a brief history and the discovery of hormonal ablation treatment. J Urol *165(6 Pt 1)*: 1859-1862, 2001. PMID: 11371867.

3 Karantanos T, Corn PG and Thompson TC: Prostate cancer progression after androgen deprivation therapy: mechanisms of castrate resistance and novel therapeutic approaches. Oncogene *32(49)*: 5501-5511, 2013. PMID: 23752182. DOI: 10.1038/onc.2013.206

4 Tannock IF, de Wit R, Berry WR, Horti J, Pluzanska A, Chi KN, Oudard S, Théodore C, James ND, Turesson I, Rosenthal MA and Eisenberger MA: TAX 327 Investigators: Docetaxel plus prednisone or mitoxantrone plus prednisone for advanced prostate cancer. N Engl J Med *351(15)*: 1502-1512, 2004. PMID: 15470213. DOI: 10.1056/NEJMoa040720

5 Tucci M, Zichi C, Buttigliero C, Vignani F, Scagliotti GV and Di Maio M: Enzalutamide-resistant castration-resistant prostate cancer: challenges and solutions. Onco Targets Ther *11*: 7353-7368, 2018. PMID: 30425524. DOI: 10.2147/OTT.S153764

6 Giacinti S, Bassanelli M, Aschelter AM, Milano A, Roberto M and Marchetti P: Resistance to abiraterone in castration-resistant prostate cancer: a review of the literature. Anticancer Res *34(11)*: 6265-6269, 2014. PMID: 25368223.

7 Liu Z, Guo BL, Gehrs BC, Nan L and Lopez RD: Ex vivo expanded human Vgamma9Vdelta2+ gammadelta-T cells mediate innate antitumor activity against human prostate cancer cells *in vitro*. J Urol *173(5)*: 1552-1556, 2005. PMID: 15821484. DOI: 10.1097/01.ju.0000154355.45816.0b

8 Uchida R, Ashihara E, Sato K, Kimura S, Kuroda J, Takeuchi M, Kawata E, Taniguchi K, Okamoto M, Shimura K, Kiyono Y, Shimazaki C, Taniwaki M and Maekawa T: Gamma delta T cells kill myeloma cells by sensing mevalonate metabolites and ICAM-1 molecules on cell surface. Biochem Biophys Res Commun *354(2)*: 613-618, 2007. PMID: 17250803. DOI: 10.1016/j.bbrc.2007.01.031

9 Mattarollo SR, Kenna T, Nieda M and Nicol AJ: Chemotherapy and zoledronate sensitize solid tumour cells to Vgamma9Vdelta2 T cell cytotoxicity. Cancer Immunol Immunother *56(8)*: 1285-1297, 2007. PMID: 17265022. DOI: 10.1007/s00262-007-0279-2

10 Yuasa T, Sato K, Ashihara E, Takeuchi M, Maita S, Tsuchiya N, Habuchi T, Maekawa T and Kimura S: Intravesical administration of gammadelta T cells successfully prevents the growth of bladder cancer in the murine model. Cancer Immunol Immunother *58(4)*: 493-502, 2009. PMID: 18682944. DOI: 10.1007/s00262-008-0571-9

11 Shimizu T, Tomogane M, Miyashita M, Ukimura O and Ashihara E: Low dose gemcitabine increases the cytotoxicity of human Vγ9Vδ2 T cells in bladder cancer cells *in vitro* and in an orthotopic xenograft model. Oncoimmunology *7(5)*: e1424671, 2018. PMID: 29721384. DOI: 10.1080/2162402X.2018.1424671

12 Dieli F, Vermijlen D, Fulfaro F, Caccamo N, Meraviglia S, Cicero G, Roberts A, Buccheri S, D'Asaro M, Gebbia N, Salerno A, Eberl M and Hayday AC: Targeting human γδ T cells with zoledronate and interleukin-2 for immunotherapy of hormone-refractory prostate cancer. Cancer Res 67(15): 7450-7457, 2007. PMID: 17671215. DOI: 10.1158/0008-5472.CAN-07-0199

13 Ojo D, Lin X, Wong N, Gu Y and Tang D: Prostate cancer stem-like cells contribute to the development of castration-resistant prostate cancer. Cancers (Basel) *7(4)*: 2290-2308, 2015. PMID: 26593949. DOI: 10.3390/cancers7040890

14 Duru N, Fan M, Candas D, Menaa C, Liu HC, Nantajit D, Wen Y, Xiao K, Eldridge A, Chromy BA, Li S, Spitz DR, Lam KS, Wicha MS and Li JJ: HER2-associated radioresistance of breast cancer stem cells isolated from HER2-negative breast cancer cells. Clin Cancer Res *18(24)*: 6634-6647, 2012. PMID: 23091114. DOI: 10.1158/1078-0432.CCR-12-1436

15 Ong CW, Kim LG, Kong HH, Low LY, Iacopetta B, Soong R and Salto-Tellez M: CD133 expression predicts for non-response to chemotherapy in colorectal cancer. Mod Pathol *23(3)*: 450-457, 2010. PMID: 20081809. DOI: 10.1038/modpathol.2009.181

16 Miyagawa F, Tanaka Y, Yamashita S and Minato N: Essential requirement of antigen presentation by monocyte lineage cells for the activation of primary human gamma delta T cells by aminobisphosphonate antigen. J Immunol *166(9)*: 5508-5514, 2001. PMID: 11313389. DOI: 10.4049/jimmunol.166.9.5508

17 Roelofs AJ, Jauhiainen M, Mönkkönen H, Rogers MJ, Mönkkönen J and Thompson K: Peripheral blood monocytes are responsible for gammadelta T cell activation induced by zoledronic acid through accumulation of IPP/DMAPP. Br J Haematol *144(2)*: 245-250, 2009. PMID: 19016713. DOI: 10.1111/j.1365-2141.2008.07435.x

18 Yuasa T, Kimura S, Ashihara E, Habuchi T and Maekawa T: Zoledronic acid – a multiplicity of anti-cancer action. Curr Med Chem *14(20)*: 2126-2135, 2007. PMID: 17691952. DOI: 10.2174/092986707781389600

19 Ashihara E, Munaka T, Kimura S, Nakagawa S, Nakagawa Y, Kanai M, Hirai H, Abe H, Miida T, Yamato S, Shoji S and Maekawa T: Isopentenyl pyrophosphate secreted from Zoledronate-stimulated myeloma cells, activates the chemotaxis of γδT cells. Biochem Biophys Res Commun *463(4)*: 650-655, 2015. PMID: 26047704. DOI: 10.1016/j.bbrc.2015.05.118

20 Nicol AJ, Tokuyama H, Mattarollo SR, Hagi T, Suzuki K, Yokokawa K and Nieda M: Clinical evaluation of autologous gamma delta T cell-based immunotherapy for metastatic solid tumours. Br J Cancer *105(6)*: 778-786, 2011. PMID: 21847128. DOI: 10.1038/bjc.2011.293

21 Durand RE and Olive PL: Resistance of tumor cells to chemo- and radiotherapy modulated by the three-dimensional architecture of solid tumors and spheroids. Methods Cell Biol *64*: 211-233, 2001. PMID: 11070841.64:211-33. DOI: 10.1016/s0091-679x(01)64015-9

22 Sharma SV, Haber DA and Settleman J: Cell line-based platforms to evaluate the therapeutic efficacy of candidate anticancer agents. Nat Rev Cancer *10(4)*: 241-253, 2010. PMID: 20300105. DOI: 10.1038/nrc2820

23 Kanwal R, Shukla S, Walker E and Gupta S: Acquisition of tumorigenic potential and therapeutic resistance in CD133+ subpopulation of prostate cancer cells exhibiting stem-cell like characteristics. Cancer Lett *430*: 25-33, 2018. PMID: 29775627. DOI: 10.1016/j.canlet.2018.05.014

24 Sohn HM, Kim B, Park M, Ko YJ, Moon YH, Sun JM, Jeong BC, Kim YW and Lim W: Effect of CD133 overexpression on bone metastasis in prostate cancer cell line LNCaP. Oncol Lett *18(2)*: 1189-1198, 2019. PMID: 31423179. DOI: 10.3892/ol.2019.10443

25 Jeter CR, Liu B, Lu Y, Chao HP, Zhang D, Liu X, Chen X, Li Q, Rycaj K, Calhoun-Davis T, Yan L, Hu Q, Wang J, Shen J, Liu S and Tang DG: NANOG reprograms prostate cancer cells to castration resistance via dynamically repressing and engaging the AR/FOXA1 signaling axis. Cell Discov *2*: 16041, 2016. PMID: 27867534. DOI: 10.1038/celldisc.2016.41

26 Rybak AP and Tang D: SOX2 plays a critical role in EGFR-mediated self-renewal of human prostate cancer stem-like cells. Cell Signal *25(12)*: 2734-2742, 2013. PMID: 24036214. DOI: 10.1016/j.cellsig.2013.08.041

27 Kregel S, Kiriluk KJ, Rosen AM, Cai Y, Reyes EE, Otto KB, Tom W, Paner GP, Szmulewitz RZ and Vander Griend DJ: Sox2 is an androgen receptor-repressed gene that promotes castration-resistant prostate cancer. PLoS One *8(1)*: e53701, 2013. PMID: 23326489. DOI: 10.1371/journal.pone.0053701

28 Rybak AP, He L, Kapoor A, Cutz JC and Tang D: Characterization of sphere-propagating cells with stem-like properties from DU145 prostate cancer cells. Biochim Biophys Acta *1813(5)*: 683-694, 2011. PMID: 21277911. DOI: 10.1016/j.bbamcr.2011.01.018

29 Toren P, Kim S, Cordonnier T, Crafter C, Davies BR, Fazli L, Gleave ME and Zoubeidi A: Combination AZD5363 with enzalutamide significantly delays enzalutamide-resistant prostate cancer in preclinical models. Eur Urol *67(6)*: 986-990, 2015. PMID: 25151012. DOI: 10.1016/j.eururo.2014.08.006

30 Zhang Z, Cheng L, Li J, Farah E, Atallah NM, Pascuzzi PE, Gupta S and Liu X: Inhibition of the Wnt/β-catenin pathway overcomes resistance to enzalutamide in castration-resistant prostate cancer. Cancer Res *78(12)*: 3147-3162, 2018. PMID: 29700003. DOI: 10.1158/0008-5472.CAN-17-3006

Received August 6, 2020
Revised August 24, 2020
Accepted August 25, 2020