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ORIGINAL RESEARCH

Tumor-infiltrating lymphocytes from human prostate tumors reveal anti-tumor reactivity and potential for adoptive cell therapy

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ABSTRACT

Advanced prostate cancer remains incurable and is the second leading cause of mortality in men. Immunotherapy based on the adoptive transfer of tumor-infiltrating lymphocytes (TIL) has demonstrated promising clinical results in patients with metastatic melanoma and lately also in other solid tumors. However, the ability to obtain TIL from patients with prostate cancer, considered poorly immunogenic, remains unknown. In this study, we investigate the feasibility of isolating and expanding TIL from primary prostate tumors. We collected tumor specimens from eight patients with diagnosed prostate adenocarcinoma undergoing radical prostatectomy and were able to successfully expand multiple autologous TIL cultures from all patients. Twenty-eight prostate-TIL cultures were further expanded using a standard rapid expansion procedure under Good Manufacturing Practice conditions. TIL cultures were phenotypically characterized for T cell subset composition, differentiation status and co-inhibitory/stimulatory markers such as PD-1, TIM-3, LAG-3, and CD28 and were found to have in general similarity to TIL obtained from patients with melanoma and lung carcinoma previously treated at our center. All analyzed TIL cultures were functional as determined by the capability to produce high level of IFNγ upon stimuli. Most importantly, co-culture assays of prostate-TIL with autologous tumors demonstrated anti-tumor reactivity.

In conclusion, these findings demonstrate that functional and anti-tumor reactive TIL can be obtained, despite the immunosuppressive microenvironment of the cancer, thus this study supports the development of TIL therapy for prostate cancer patients.

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Introduction

Prostate cancer (PCa) is the second most commonly diagnosed cancer and the second leading cause of cancer mortality in men.[1] Patients with localized PCa are mostly treated with surgery or radiotherapy. Since prostate cancer is typically hormone-dependent the main therapy for patients with locally advanced and metastatic disease is androgen-deprivation therapy.[1-3] This treatment can provide remission for a few years, but subsequently disease often progresses to castrate-resistant prostate cancer (CRPC). While various treatment options for CRPC are available and demonstrated significant improvement in overall survival, metastatic PCa is associated with poorer prognosis and remains incurable,[4,5] emphasizing the need in developing novel treatment strategies.

Targeting the immune system to elicit an anti-tumor response and eradicate tumor cells represents a promising strategy for the development of anti-cancer treatments. Sipuleucel-T, an autologous vaccine is the first therapeutic cancer vaccine and currently the only FDA-approved immune therapy for PCa.[6] Yet, in comparison with other cancer types, immunotherapy approaches in prostate cancer resulted in a high degree of failure and still remain a challenge in the immunotherapy field.[4,7,8]

Immunotherapy based on adoptive cell therapy (ACT) of TIL has proven to be highly effective in patients with metastatic melanoma. Others and we could show that TIL therapy yields response rates of around 40% with durable complete response rates of 10% to 20% in metastatic melanoma patients.[9-14] In addition, we recently reported the pre-clinical production and evaluation of TIL for adoptive cell therapy in lung cancer patients to support the implementation of TIL ACT for these patients.[15]

Ex vivo TIL production requires several steps which start with the processing of surgically resected tumor tissue and continues with the expansion of lymphocytes in interleukin (IL) −2 containing medium for 2 to 4 weeks to establish a TIL culture ("pre-REP"). In the next step, TIL are massively expanded for 14 days to large numbers using a Rapid Expansion Procedure (REP) with anti-CD3 antibody, IL-2, and irradiated feeder cells. On day 14 of REP, TIL are washed and prepared for infusion.

The PCa microenvironment, considered to be mostly immunosuppressive, may contribute to the limited success of

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immunotherapy-based trials in PCa patients so far. The high heterogeneity of the tumor composition adds one more level of complexity.[16,17] In the late stage of the disease, alterations in DNA damage repair mechanisms further increase genomic instability and mutational divergence in patients.[17] Although prostate cancer accounts for a cancer with relative low mutation rate (~0.9 mutations/Mb)[18,19] several studies have shown that even cancers with lower mutational load can be targeted by T cells directed against mutation-derived neo-antigens.[20] The high heterogeneity of PCa and the existence of patient-specific tumor mutations provide the rational in developing personalized immunotherapies that target specific tumor neo-antigens of each patient. Design of personalized treatments, such as neo-antigen vaccines or T cell therapy, are likely to have a clinical benefit in PCa patients.

Using mainly immunohistochemistry, only a few studies reported the existence of prostate-infiltrating immune cells in normal and tumor tissues until today.[21-24] These studies proposed that prostate cancer-derived TIL are nonfunctional and refractory to stimulation.[24-26] However, isolation and expansion of TIL cultures from PCa patients ("prostate-TIL") and the analysis of functionality and anti-tumor reactivity of the expanded TIL have not been investigated yet.

In the current study, we isolated and expanded TIL from patients diagnosed with prostate adenocarcinoma under Good Manufacturing Practice (GMP) conditions with clinically compliant reagents. TIL were phenotypically characterized and functionality was assessed. TIL were further exposed to their counterpart autologous tumor lines to examine anti-tumor reactivity.

Material and methods

Patients and patient-derived tissues

Eight prostatic cancer patients with high tumor burden (>15 mm^3) undergoing radical prostatectomy were included to the study. The fresh tissue was cut in the middle and samples were taken from carcinoma suspected areas according to Wheeler and Lebovitz with a punch biopsy instrument.[27] In seven more patients, fresh tumor tissue was collected from transrectal ultrasound–guided prostate (TRUS-Bx) biopsies. The fresh tissue samples were transferred to a GMP facility for processing.

Generation and expansion of TIL culture

Prostate punches and TRUS-Bx biopsies were cut into multiple fragments of about 0.5–1 mm^3 of size. Each fragment was placed into one well of a 24-well plate with 2 ml complete medium (CM) comprised of RPMI 1640 medium (Gibco, Thermo Fisher Scientific, Waltham, MA) containing 10% heat-inactivated human serum (Valley Biomedical, Winchester, VA or Gemini Bio, West Sacramento, CA), 25 mmol/l HEPES pH 7.2 (Gibco), 50 µg/ml gentamycin (Gentamicin IKA, Teva, Israel), 100 U/ml penicillin and 100 mg/ml streptomycin (Gibco) and supplemented with 3,000 IU/ml recombinant human IL-2 (Proleukin, Novartis Pharma, Germany). Prostate-TIL were allowed to extravasate from the fragments. Half of the media was replaced every 2 to 3 days. Following the establishment of TIL cultures, a standard 14-days Rapid Expansion Procedure (REP) was initiated by stimulating prostate-TIL with 30 ng/ml anti-CD3 antibody MACS GMP CD3 pure (clone OKT-3; Miltenyi Biotech, Germany), 3,000 IU/ml IL-2, and irradiated peripheral blood mononuclear cells from non-related donors as feeder cells (5000 rad, 100:1 ratio between feeder cells and TIL) in 50% CM, 50% AIM-V medium (Invitrogen, Thermo Fisher Scientific, Waltham, MA) (for details see Besser MJ[28]; Itzhaki O[29]). REP was performed in T25 flasks or 24-well plates.

Isolation and establishment of primary prostate cancer cultures

Primary prostate cancer cultures were initiated as described for TIL culture. Whenever adherent monolayer of epithelial-shaped cells propagated from the fragments, cells were re-suspended in Keratinocyte-SFM Medium Kit containing L-glutamine (Cat #17005042, Gibco), and supplemented with bovine pituitary extract (BPE) 25 µg/ml and human epithelial growth factor (EGF) 10 ng/ml and transferred to 6-well culture plates coated with collagen I, Rat Tail (Thermo Scientific #A1142801) or poly-D lysine (Corning #356413).

Flow cytometry

Prostate-TIL cultures were stained using mouse anti-human antibodies against the following membrane molecules: CD3 (VioBlue; Miltenyi Biotech), CD4 (FITC; BioLegend), CD8 (PE-Cy7; BioLegend), CD137 (APC; clone: 4B4-1; BioLegend), CD134 (FITC; clone: Ber-ACT35; BioLegend), PD-1 (FITC; clone: EH12.2H7; BioLegend), TIM-3 (PE; R&D), CD25 (APC; eBioscience), CD28 (APC; eBioscience), LAG-3 (Per-CP-Vio770; Miltenyi Biotech), CD45RA (APC-Vio770; Miltenyi Biotech) CCR7 (FITC; BioLegend), CCR2 (APC; Biolegend), CCR4 (PE; Biolegend), CCR5 (alexa488; Biolegend), CCR7 (PerCP, Biolegend), CXCR2 (PE; Biolegend) and CXCR3 (APC; Biolegend). TIL were washed and re-suspended in cell staining buffer (BioLegend). Cells were incubated for 30 min with the antibodies on ice, washed in buffer, and measured using MACSQuant flow cytometer (Miltenyi Biotech). Samples were analyzed using FlowJo software. (FlowJo LLC, Ashland, OR).

Primary prostate cancer cultures were stained with surface marker CD90 (clone F15-42-1, FITC, Abcam), HLA-A, B, C (PE-Cy7; BioLegend), HLA-DP, DQ, DR (PerCP; Biolegend), PDL-1 (PE; Biolegend), CD86 (FITC; Biolegend), CD80 (APC; Biolegend) and the following intracellular antibodies: Cytokeratin 5 (clone EP1601Y, Alexa Fluor® 488, Abacm), Cytokeratin 8 (clone EP1628Y, Alexa Fluor® 647, Abcam), PSMA (clone GCP-05, PE, Abcam), Androgen Receptor (clone ER179(2), Alexa Fluor® 594, Abacm), followed by fixation and permeabilization (eBioscience Intracellular Fixation & Permeabilization Buffer kit) according to manufacturing instructions and analyzed using FlowJo software.

Functionality

To determine functionality, 1x10e5 TIL were stimulated with 10 µg/ml MACS GMP CD3 pure antibody overnight and IFNγ levels were determined by ELISA according to the manufacturer's instructions (BioLegend, San Diego, CA). Measurements were performed in triplicates. To determine intracellular IFNγ levels

2x10e5 TIL were stimulated with 10 μg/ml MACS GMP CD3 pure antibody for 2 h and brefeldin A was added for an additional 2 h according to the manufacturer's instructions. Flow cytometry was performed by the addition of CD8 antibody (PE-Cy7 conjugated; Biolegend) followed by fixation and permeabilization (eBioscience Intracellular Fixation & Permeabilization Buffer kit) and addition of IFNγ antibody (APC conjugated; clone 4S. B3; Invitrogen). Samples were analyzed using FlowJo software. Measurements were performed in triplicates.

Anti-tumor reactivity

1x10e5 TIL were co-cultured with primary prostate cancer cultures in 96-well plates for 18 h at different E:T ratio (as indicated). The anti-tumor reactivity of TIL was determined by IFNγ ELISA or CD137 and CD134 flow cytometry as described above. For CD107a expression, monensin (3μM) and brefeldin A (2μM) were added to each well after 1 h of co-culture. After 16 h cells were washed with staining buffer, and then surface stained with anti CD3 and anti-CD8 antibodies for 30 min. Following, fixation and permeabilization, the cells were stained with CD107a (FITC; BD) for 30 min, washed and analyzed using FlowJo software. For MHC I blocking experiments target cells were pre-treated with 20 μg/ml of anti-human HLA-A, B, C antibody (clone W6/32; Biolegend), 1 h before the addition of the TIL.

TCR analysis

T cell clonality was analyzed by detection of TCR-γ gene rearrangement as described in the BIOMED-2 protocol.[30] Briefly, DNA samples were extracted from four pre-REP cultures and their corresponding post-REP cultures. For each DNA sample, four sets of fluorescent-labeled primers for the forward V heavy chain (V9/2, Vfl, V11, and V10/2) were used. The PCR products were analyzed by Genescan analysis.

Statistical analysis

Significance of variation between groups was evaluated using a non-parametric two-tailed Student's *t* test. Test for differences between proportions was performed using two-sided Fisher's exact test with $p \leq 0.05$ considered significant.

Results

Establishment of TIL from prostate cancer-derived tissues

Primary prostate tumor specimens were obtained from eight patients following radical prostatectomy (patients' characteristics see **Supplementary Table S1**). The patients enrolled to this study were with high tumor burden (>~15 mm^3) by sonography or MRI, as well as with grossly visible tumors. The average age of the patients was 68 years (range 61–79

Table 1. TIL isolation and expansion from prostate tumor punches.

Patient	Punch	No. of fragments initiated	No. of fragments grown TIL	Total no. of TIL per patient
	Left (L1)	9	0	1.30E+05
PS-001	Left (L2)	8	0	
	Right (R1)	6	2	
	Right (R2)	6	1	
	Total 4	**29**	**3**	
	Left (L1)	6	5	1.01E+06
PS-002	Left (L2)	6	5	
	Right (R1)	6	6	
	Right (R2)	6	6	
	Total 4	**24**	**22**	
	Left (L)	5	0	1.80E+05
PS-003	Right (R)	6	5	
	Center (C)	3	0	
	Anterior (A)	4	3	
	Total 4	**18**	**8**	
	Left (L)	8	8	4.70E+05
PS-004	Right (R)	8	6	
	Center (C)	6	2	
	Total 3	**22**	**16**	
	Left (L)	8	2	1.60E+05
PS-005	Right (R)	8	1	
	Center (C)	6	1	
	Anterior (A)	6	0	
	Total 4	**28**	**4**	
	Left (L)	12	9	2.89E+06
PS-006	Right (R)	8	6	
	Center (C)	6	4	
	Anterior (A)	8	8	
	Total 4	**34**	**27**	
	Left (L1)	4	1	7.19E+06
PS-007	Left (L2)	6	3	
	Right (R1)	6	5	
	Right (R2)	5	3	
	Total 4	**21**	**12**	
	Left (L1)	8	7	2.91E+06
PS-008	Left (L2)	6	5	
	Right (R1)	6	6	
	Right (R2)	5	1	
	Total 4	**25**	**19**	
Total	**31**	**201**	**111 (55%)**	

years), the average Gleason score 7 and the average serum PSA level 6.2 ng/ml (range 1.4 ng/ml to 7.6 ng/ml). Six patients had acinar adenocarcinoma (PS-001 to 004 and PS-007-008), one patient ductal adenocarcinoma (PS-006) and one patient had mixed acinar and ductal adenocarcinoma (PS-005). Samples for TIL processing were obtained from 3 to 4 punches of different areas in the tumor. The size of each punch was only approximately 0.03 cm^3 **(Supplementary Fig. S1A)**. Each punch was named according to the location it was taken from (L = left, R = right, C = center, A = anterior). Sometimes more than one punch was taken from the same location (e.g. L1, L2) (Table 1). The tissues were evaluated by a pathologist. Prostatic malignant epithelial cells were clearly identified by morphology and demonstrated focal lymphocyte infiltration in the resected tissues (**Supplementary Fig. S2)**. T and B cells were distinguished by immunohistochemistry (**Supplementary Fig. S3)**.

TIL establishment and expansion were performed with clinically compatible reagents under GMP conditions. Each of the 3 to 4 punch biopsies was cut into three to twelve 0.5–1 mm^3 fragments, resulting in a total of 18 to 34 fragments per patient, and placed in complete media containing 3,000 IU/ml IL-2. TIL cultures were initiated in 24-well plates by transferring one fragment into one well. Within 2 to 4 weeks TIL outgrowth was observed from 111 of 201 (55%) initiated TIL cultures and from at least three fragments of each patient, demonstrating the feasibility to generate TIL from radical prostatectomy biopsies (Table 1).

Altogether 1.3x10e5 – 7.19x10e6 (average of 1.87x10e6 ± 2.45x10e6) TIL were established per patient within 2 to 4 weeks from three to four 0.03 cm^3 small punches. In comparison, the average volume of melanoma specimens we typically received for clinical TIL production is about 250-times bigger (average volume 24 ± 50 cm^3). A prostate biopsy of such a size could theoretically yield 3.8x10e8 ± 4.87x10e8 TIL, demonstrating the potential of prostate tumor-derived tissue as a source for TIL establishment.

The phenotype of eleven pre-REP TIL cultures derived from patients PS-007 and 008 were analyzed by flow cytometry. Pre-REP TIL analysis showed that the majority of the cells were CD3 + T cells (88 ± 5%, range 79%-96%) while only 4 ± 3% were CD56+ CD3-NK cells. The results of a detailed phenotype analysis, including CD4, CD8, PD-1, LAG-3, TIM-3, CD25, CD28 subsets, and differentiation status are shown in Table 2.

In order to estimate the proliferative capacity of prostate-TIL, small-scale rapid expansion procedures (REP) were initiated with irradiated PBMC feeders, soluble anti-CD3 antibody and IL-2 Briefly, 28 TIL cultures obtained from single fragments or a pool of fragments (2 to 6 fragments from the same punch) were expanded for 14 to 15 days. Morphology of the prostate-derived TIL cultures **(Supplementary Fig. S1B)** was similar to the ones observed in TIL derived from melanoma biopsies (melanoma-TIL, n = 103, partly published in Besser MJ, et al.[10]) and Non-Small Cell Lung Carcinoma biopsies (NSCLC-TIL, n = 15),[15] which were obtained from patients previously treated in our center. Growth curves of 28 TIL cultures, derived

Table 2. Phenotypic analysis of pre-REP prostate-TIL. Phenotype analysis of 11 pre-REP TIL cultures derived from two prostate cancer patients. Cells were gated on viable, singlet CD3 T cells. TN (naïve), CD3+CD45RA+CCR7+; TCM (central memory), CD3+CD45RA−CCR7+; TEM (effector memory), CD3+CD45RA−CCR7−; TEMRA (effector), CD3+CD45RA+CCR7−.

TIL culture #	Patient	CD56+ CD3-	CD3+	CD8+ CD3+	CD4+ CD3+	PD1+ CD3+	PD1+ CD8+	PD1+ CD4+	LAG3+ CD3+	TIM-3+ CD3+	CD25+ CD3+	CD28+ CD3+	CD28+ CD8+	CD28+ CD4+	TN	TCM	TEMRA	TEM
1	PS-007 a	7.4	91.9	9.0	91.0	14.2	0.7	13.5	36.0	59.3	25.4	93.5	8.1	85.4	3.1	29.9	1.1	65.9
2	PS-007 b	8.0	89.1	6.8	93.2	39.8	0.6	39.2	45.0	31.7	36.8	86.2	6.4	79.8	1.1	20.6	0.9	77.4
3	PS-007 c	8.3	90.8	16.3	83.7	18.1	2.0	16.2	33.2	32.4	31.8	84.3	11.8	72.4	0.2	6.2	1.7	92.0
4	PS-007 d	5.7	92.6	8.5	91.5	19.2	1.8	17.4	28.5	30.3	48.7	89.3	6.8	82.5	0.5	11.0	0.8	87.8
5	PS-007 e	4.3	90.4	6.3	93.7	67.5	1.3	66.2	11.5	29.4	22.3	95.2	11.1	84.1	4.9	35.8	1.6	57.7
6	PS-007 f	0.0	82.7	19.4	80.6	40.5	5.5	35.0	5.2	16.3	45.5	75.1	13.7	61.4	1.3	5.7	7.9	85.1
	PS-007 Average	**5.6**	**89.6**	**11.0**	**88.9**	**33.2**	**2.0**	**31.3**	**26.6**	**33.2**	**35.1**	**87.3**	**9.6**	**77.6**	**1.8**	**18.2**	**2.3**	**77.7**
7	PS-008 a	0.0	96.2	2.1	97.9	86.5	0.5	86.0	5.8	37.0	19.9	98.1	0.5	97.6	0.0	0.2	1.5	98.3
8	PS-008 b	1.4	79.3	30.0	70.0	66.9	15.3	51.6	26.9	29.2	43.5	58.1	13.3	44.8	1.7	14.2	10.6	73.5
9	PS-008 c	3.3	90.8	19.2	80.8	40.4	5.4	35.0	4.6	20.3	7.1	70.5	11.3	59.2	2.3	14.2	11.8	71.8
10	PS-008 d	0.0	82.1	34.9	65.1	31.9	4.6	27.3	11.8	41.8	13.1	25.6	4.1	21.5	0.8	2.0	41.4	55.8
11	PS-008 e	1.2	81.0	19.0	81.0	56.2	6.3	49.9	15.7	35.0	30.9	57.3	10.0	47.3	1.2	8.6	6.6	83.6
	PS-008 Average	**1.2**	**85.9**	**21.0**	**79.0**	**56.4**	**6.4**	**50.0**	**12.9**	**32.7**	**22.9**	**61.9**	**7.8**	**54.1**	**1.2**	**7.8**	**14.4**	**76.6**
Average		**3.8**	**88.0**	**15.2**	**84.8**	**42.9**	**3.8**	**39.0**	**20.9**	**33.0**	**30.0**	**76.7**	**8.9**	**67.8**	**1.6**	**13.9**	**7.4**	**77.2**
S.D		**3.2**	**5.4**	**9.9**	**9.9**	**22.3**	**4.2**	**21.5**	**13.6**	**10.7**	**12.9**	**21.1**	**3.9**	**21.6**	**1.4**	**10.8**	**11.4**	**13.1**

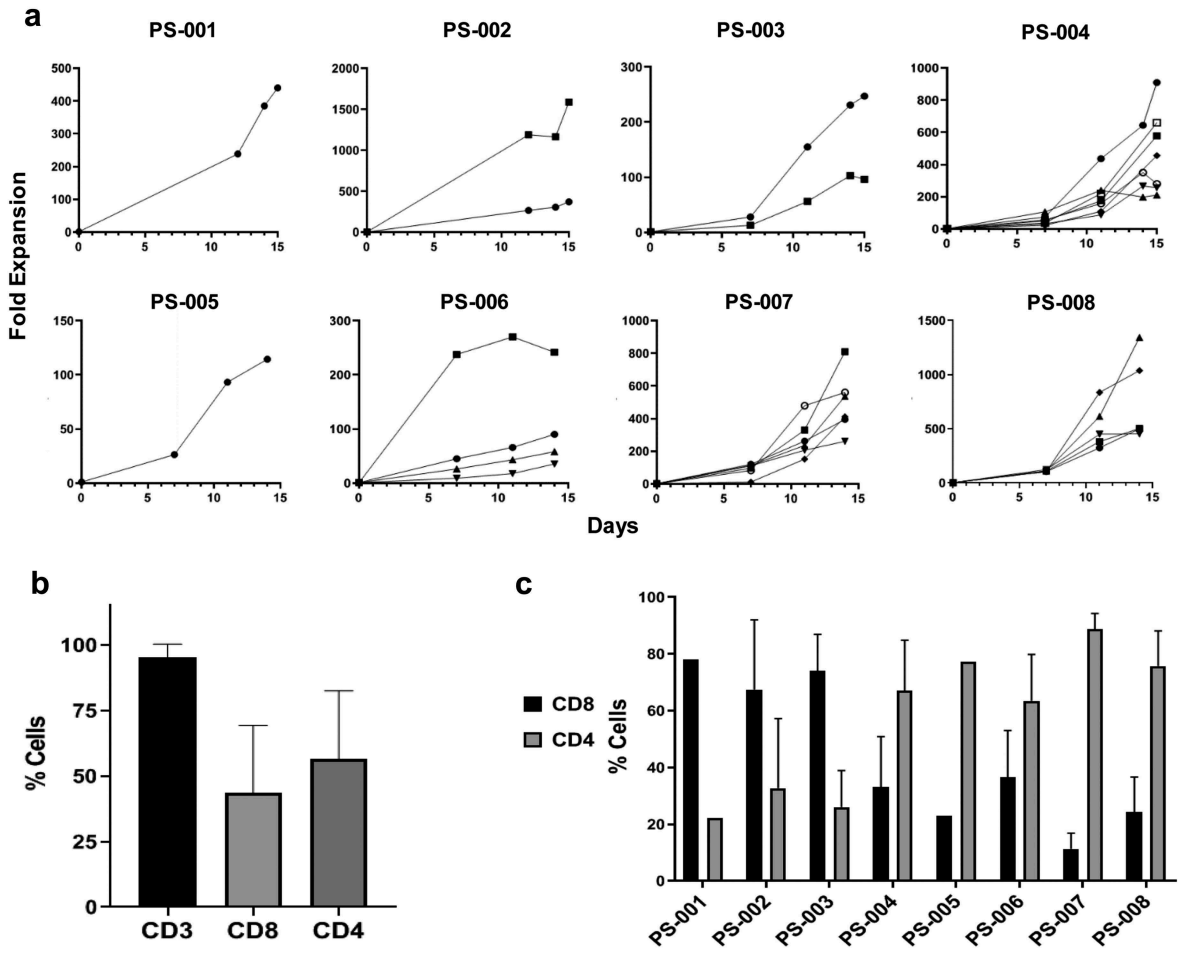

Figure 1. Expansion and phenotype analysis of TIL cultures from radical prostatectomy specimens. (A) Fold expansion during rapid expansion procedure (REP) of one or multiple TIL cultures obtained from patients PS-001-008. Frequency of CD3, CD4, and CD8 in 28 post-REP TIL cultures derived from PS-001-008 (B), and for each patient (C).

from the eight patients, are shown in Figure 1a. TIL obtained from different areas of the same tumor demonstrated diverse fold expansion indicating the heterogeneity of the TIL (Table 3). An average fold expansion of 475 ± 376 (range 35–1588) was achieved, which is lower compared with melanoma-TIL (1081 ± 493 fold, n = 103, $p < .001$) and NSCLC-TIL (1121 ± 349, n = 15, $p < .001$), but high enough to comply with ongoing clinical ACT trials, which demand a minimum cell number of 5x10e9 TIL (see NCT00287131, NCT02278887), which can be reached with an average of only 11x10e6 TIL at REP initiation.

Despite the fact that sufficient cell numbers for therapy can be reached, we evaluated the possibility to perform a second round of REP (REP-2). Six TIL cultures were rapidly expanded an additional time and underwent a further expansion of 100-fold ± 68 **(Supplementary Table S2)**.

Prostate-TIL clonality was evaluated by comparing the TCR-γ rearrangement before and after REP. In general, the TCR analysis demonstrated that all the types of clonal expansion (poly, oligo, and mono-clonal) could be detected on pre-REP TIL, as well as on post-REP TIL. The specific changes for four pre and post-REP cultures are shown in **Supplementary Fig. S4** and summarized in **Supplementary Table S3**. We could not detect a preferential type of clonal expansion during REP.

Phenotypic analysis of prostate-TIL revealed general similarities in the characteristics compared with melanoma-TIL and NSCLC-TIL

Twenty-eight post-REP TIL derived from eight patients cultures were analyzed for CD3, CD4, and CD8 expression. The average frequency was 95 ± 5% CD3 T cells, including 43 ± 26% (range 4–85%) CD8 T cells and 57 ± 26% (range 15–96%) CD4 T cells (Figure 1b). The ratio of CD8:CD4 T cells for each patient is shown in Figure 1c. The CD8 frequency in post-REP prostate-TIL was similar to melanoma-TIL (59 ± 25%; n = 103; $p = .092$) and NSCLC-TIL (44 ± 30%; n = 15; $p = .97$) (Table 4).

All 28 post-REP prostate-TIL were further characterized for expression of inhibitory molecules (PD-1, LAG-3, TIM-3), co-stimulatory molecules (CD25, CD28) and differentiation markers (CD45RA, CCR7). Representative flow cytometric plots are shown in **Supplementary Fig. S5** and the data is summarized in Table 5. The average percentage of PD1 on CD3 T cells was 47 ± 17% with a distribution of 17 ± 15% on CD8 and 30 ± 17%

Table 3. Rapid expansion of prostate-TIL cultures. Twenty-eight TIL cultures from eight patients underwent small-scale rapid expansion procedure (REP). Cell numbers at initiation (REP day 0) and at completion (REP day 14/15) are shown.

TIL culture No.	Patient	Punch/Frag.	REP day 0 no. of cells	REP day 14/15 no. of cells	Fold expansion
1	PS-001	R2/F6	3.0E+04	1.32E+07	440
2	PS-002	L/F2,5	1.6E+04	2.54E+07	1588
3		L/F3,4	7.0E+04	2.59E+07	370
4	PS-003	R/F1-5	1.0E+05	2.47E+07	247
5		A/F1,3,4	8.0E+04	8.00E+06	100
6	PS-004	L/F1-3	1.0E+05	9.10E+07	910
7		L/F7-8	5.0E+04	2.89E+07	578
8		R/F1-3	1.0E+05	2.10E+07	210
9		R/F4	3.0E+04	7.70E+06	257
10		R/F7-8	5.0E+04	2.28E+07	456
11		C/F2	3.0E+04	8.40E+06	280
12		C/F5	3.0E+04	1.98E+07	660
13	PS-005	R/F3	3.00E+04	3.43E+06	114
14	PS-006	L/F7,10,12	4.0E+05	3.61E+07	90
15		R/F5,7	6.0E+04	1.45E+07	242
16		C/F6	1.6E+05	9.30E+06	58
17		A/F5-8	1.5E+06	5.30E+07	35
18	PS-007	R1/F2	5.0E+04	2.0E+07	395
19		R1/F5	5.0E+04	4.1E+07	810
20		R1/F3+4	7.5E+04	4.2E+07	560
21		L2/F3	7.5E+04	4.0E+07	537
22		L2/F4	1.0E+05	2.6E+07	261
23		L2/F2	5.0E+04	2.1E+07	411
24	PS-008	L1/F3-4	1.2E+05	6.0E+07	513
25		L1/F1-2, 5-6, 7-8	5.0E+04	2.5E+07	502
26		L2/F6	2.5E+04	3.4E+07	1344
27		R1/F2	1.8E+05	5.1E+07	286
28		R1/F1+5	5.0E+04	5.2E+07	1038
Average				**2.9E+07**	**475**
SD				1.9E+07	376

Table 4. Phenotype analysis of post-REP TIL derived from prostate carcinoma, melanoma (Mel) and lung carcinoma. *ND, not determined.*

	Prostate TIL n = 28	Mel TIL n = 103	Lung TIL n = 15
CD3+CD8+	43 ± 26%	59 ± 25%	44 + 30%
CD3+CD4+	57 ± 26%	41 ± 25%	56 ± 30%
PD1+CD3+	47 ± 17%	42 ± 25%	57 ± 25%
CD28+CD3+	74 ± 18%	58 ± 25%	37 ± 19%
CD45RA-CCR7–(TEM)	80 ± 9%	97 ± 3	*ND*

on CD4 T cells. The percentage of PD-1 positive prostate-TIL was similar to melanoma-TIL (42 ± 25%, n = 103, p = .597) and NSCLC-TIL (57 ± 25%, n = 15; p = .393) (Table 4). Noteworthy, 74 ± 18% (range 26–98%) of prostate-TIL expressed the co-stimulatory molecule CD28, which is comparable with melanoma-TIL (58 ± 25%, n = 103, p = .077), but significantly higher than the level detected on NSCLC-TIL (37 ± 19%, n = 15, p = .005). CD28+ expression was found on CD8 (26 ± 25%, range 2%-82%), as well as on CD4 (48 ± 25%, range 11–96%) prostate-TIL (Table 5). In addition, 38 ± 25% (range 21–87%) of the cells expressed the activation marker CD25, which was mainly expressed on CD8 T cells (26 ± 28%). The differentiation status of TIL cultures was defined based on the expression of CD45RA in combination with CCR7. The majority of the T cells (80 ± 9%) were effector memory T cells (TEM, CD45RA-CCR7-), while 10 ± 4% were terminal effector memory (TEMRA, CD45RA+CCR7-) and 9 ± 7% central memory T cells (TCM, CD45RA-CCR7+). In comparison, melanoma-TIL differentiated almost exclusively to TEM following REP (97 ± 3%, n = 56; p < .0001) (Table 4).

In-vitro establishment of primary prostate cancer cultures

The establishment of primary prostate cancer (PCa) cultures is considered technically challenging. Most of the existing prostate cancer lines were obtained from prostate tumor metastases, while only a few reports have shown the success of growing cultures from primary prostate cancer.[31-33]

From patient PS-002 onwards, we tried to establish primary prostate cancer cultures. Of 172 initiated fragments, we observed an adherent monolayer of outgrowing cells with typical morphological characteristics of epithelial cells in 85 fragments (49%) (**Supplementary Fig. S6**). Interestingly, 55 of 85 (64%) epithelial cell containing cultures gave also rise to TIL **(Supplementary Table S4)**. Primary prostate cancer cultures were grown with keratinocyte serum-free medium (KSFM) supplemented with epidermal growth factor (EGF) and bovine pituitary extract (BPE) combined with poly-D-lysine or collagen type I coated plates. Prostate cancer cultures were established from seven of seven patients (PS-002-008). The tumor cells grew for 3 to 4 passages. Phenotype characterization of the cells showed the absence of the fibroblasts marker CD90 and high expression of the epithelial markers cytokeratin 5 (CK5, basal marker, 81%) and cytokeratin 8 (CK8, luminal marker, 91%). In most PCa cultures, CK5 and CK8 were co-expressed (above 80%) indicating an intermediate phenotype.[30] Prostatic origin was verified by the expression of prostate-specific membrane antigen (PSMA) and androgen receptor (AR). Representative flow cytometric plots are illustrated in Figure 2a and data summarized for all patients in Figure 2b–d. Due to the presence of tumor cells in the resected tissues **(Supplementary Fig. S2)**, one can assume that the obtained cultures are carcinoma cells. High CK5 and CK8 expressions were described as markers of malignant cells[28,34,35] although no specific markers exists, which definitively distinguish between tumor cells and non-tumor prostate epithelium.

Three PCa cultures derived from patients PS-002 and 008 were further analyzed for expression of human leukocyte antigen (HLA) class 1 and class 2, the co-stimulatory ligands CD80 and CD86 and the co-inhibitor ligand PD-L1 (Figure 2e–g). HLA-A, B, C was highly expressed on all cultures, while HLA-DP, DQ, DR expression was lower, but comparable to HLA-DP, DQ, DR expression on melanoma cultures (n = 4, p = .075) (Figure 2f).

Prostate-derived-TIL are functional and anti-tumor reactive

Functionality of post-REP TIL was evaluated by stimulating TIL with an anti-CD3 antibody followed by IFNγ ELISA. Twelve cell cultures, derived from six patients, were analyzed and the average secretion of IFNγ was 45,571 ± 22,896 pg/ml (range 13,703–86,372 pg/ml) (Figure 3a), demonstrating that all analyzed TIL cultures were functional and could secret very high levels of IFNγ. For comparison, we measured a NSCLC-TIL culture, which secreted 28,273 pg/ml. Intracellular FACS analysis of IFNγ following anti-CD3 stimulation showed that a high fraction of the T cells had the ability to secrete IFNγ (57% ± 18, range 29%-76) including CD8 (30 ± 17%, range 8%-64%) and CD4 (27 ± 16%, range 6%-57%) (Figure 3b,c). After confirming TIL functionality,

Table 5. Phenotypic analysis of post-REP prostate-TIL. Phenotype analysis of 28 post-REP TIL cultures derived from eight prostate cancer patients. Cells were gated on viable, singlet CD3 T cells. TN (naïve), CD3+CD45RA+CCR7+; TCM (central memory), CD3+CD45RA−CCR7+; TEM (effector memory), CD3+CD45RA−CCR7−; TEMRA (effector), CD3+CD45RA+CCR7−.

TIL #	Patient	CD8+ CD3+	CD4+ CD3+	CD3+ PD1+	PD1+ CD8+	PD1+ CD4+	LAG-3+ CD3+	TIM-3+ CD3+	CD25+ CD3+	CD28+ CD3+	CD28+ CD8+	CD28+ CD4+	TN	TCM	TEMRA	TEM
1	**PS-001**	**74.8**	**25.2**	**24.7**	**18.9**	**5.8**	**32.8**	**58.9**	**78.7**	**90.2**	**65.6**	**24.6**	**0.8**	**2.6**	**11.1**	**85.6**
2	PS-002 a	50.0	50.0	23.6	11.9	11.7	27.8	75.0	53.5	87.0	42.1	44.9	3.5	14.8	5.3	76.4
3	PS-002 b	84.7	15.3	21.4	18.2	3.2	31.8	84.3	86.6	92.6	81.7	10.9	0.5	3.4	20.3	75.7
	PS-002 Avg.	**67.4**	**32.7**	**22.5**	**15.1**	**7.4**	**29.8**	**79.7**	**70.1**	**89.8**	**61.9**	**27.9**	**2.0**	**9.1**	**12.8**	**76.1**
4	PS-003 a	83.1	16.9	74.2	61.1	13.1	31.4	27.8	78.0	26.3	15.5	10.8	0.4	7.1	1.2	91.3
5	PS-003 b	64.9	35.1	70.4	38.2	32.2	14.6	19.7	12.7	64.1	41.0	22.4	6.1	37.0	5.9	51.0
	PS-003 Avg.	**74.0**	**26.0**	**72.3**	**49.7**	**22.7**	**23.0**	**23.8**	**45.3**	**45.2**	**28.3**	**16.6**	**3.2**	**22.1**	**3.5**	**71.2**
6	PS-004 a	38.8	61.2	66.7	26.8	39.9	17.6	19.0	42.8	62.4	13.4	49.0	0.9	7.5	11.0	80.6
7	PS-004 b	18.0	82.0	74.9	14.8	60.1	7.8	22.1	21.0	65.8	4.4	61.4	0.5	5.1	3.4	91.0
8	PS-004 c	59.2	40.8	47.7	23.8	23.9	22.6	35.1	3.8	48.6	24.0	24.6	6.8	21.8	23.8	47.6
9	PS-004 d	53.3	46.7	52.5	23.4	29.1	18.1	11.3	9.2	81.9	39.2	42.7	2.2	20.1	16.9	60.9
10	PS-004 e	22.6	77.4	30.0	12.4	17.6	10.2	6.4	3.9	43.4	3.3	40.1	1.3	10.1	29.3	59.2
11	PS-005 f	26.0	74.0	63.5	15.6	47.9	24.0	33.2	20.9	72.3	7.9	64.4	0.3	9.9	4.9	84.8
12	PS-004 g	13.1	86.9	41.7	5.4	36.3	15.1	9.9	12.6	56.5	4.8	51.7	0.6	5.7	9.6	84.2
	PS-004 Avg.	**33.0**	**67.0**	**53.9**	**17.5**	**36.4**	**16.5**	**19.6**	**16.3**	**61.6**	**13.9**	**47.7**	**1.8**	**11.4**	**14.1**	**72.6**
13	**PS-005**	**22.9**	**77.1**	**51.0**	**4.6**	**46.4**	**14.8**	**8.5**	**15.2**	**59.0**	**2.6**	**56.4**	**1.3**	**13.2**	**11.6**	**73.9**
14	PS-006 a	56.7	43.3	58.5	35.6	22.9	10.6	25.0	59.6	71.2	35.2	36.0	2.9	5.5	26.0	65.6
15	PS-006 b	38.8	61.2	17.4	5.4	12.0	14.9	15.9	14.3	81.9	25.0	56.9	5.8	17.0	15.9	61.3
16	PS-006 c	16.9	83.1	54.6	2.6	52.0	41.3	47.5	19.6	67.7	8.8	58.9	1.5	5.3	5.1	88.2
17	PS-006 d	33.9	66.1	44.7	5.4	39.3	19.9	23.1	13.1	53.2	9.1	44.1	1.9	13.4	4.1	80.6
	PS-006 Avg.	**36.6**	**63.4**	**43.8**	**12.2**	**31.6**	**21.7**	**27.9**	**26.6**	**68.5**	**19.5**	**49.0**	**3.0**	**10.3**	**12.8**	**73.9**
18	PS-007 a	9.8	90.3	35.9	2.1	33.8	10.4	82.9	42.7	96.1	7.0	89.1	0.4	0.4	1.1	98.2
19	PS-007 b	10.5	89.5	19.0	1.5	17.5	6.0	69.9	30.1	95.4	7.4	88.0	0.1	0.2	3.1	96.6
20	PS-007 c	20.4	79.6	48.1	6.2	41.9	8.5	82.1	39.4	89.9	12.4	77.5	0.1	0.3	3.2	96.4
21	PS-007 d	9.6	90.4	41.8	3.0	38.8	23.2	85.8	58.9	92.3	5.6	86.7	0.5	0.5	0.6	98.3
22	PS-007 e	3.7	96.3	88.7	2.1	86.6	23.8	82.0	45.1	98.5	2.4	96.1	0.3	2.2	7.1	90.4
23	PS-007 f	13.9	86.1	40.1	5.4	34.7	2.9	31.7	10.1	92.3	9.3	83.0	0.1	0.5	6.2	93.2
	PS-007 Avg.	**11.3**	**88.7**	**45.6**	**3.4**	**42.2**	**12.5**	**72.4**	**37.7**	**94.1**	**7.4**	**86.7**	**0.2**	**0.7**	**3.6**	**95.5**
24	PS-008 a	8.4	91.6	94.4	6.2	88.2	9.4	33.5	12.6	94.7	3.6	91.1	0.1	0.4	1.7	97.9
25	PS-008 b	20.0	80.0	48.8	11.6	37.2	3.1	25.2	11.2	90.8	11.5	79.3	0.1	0.3	4.3	95.3
26	PS-008 c	20.0	80.0	34.4	7.7	26.7	5.3	40.1	12.5	88.5	10.8	77.7	0.0	0.3	9.3	90.4
27	PS-008 d	32.9	67.1	63.6	19.4	44.2	8.3	33.4	19.7	77.2	14.1	63.1	0.1	0.9	14.2	84.8
28	PS-008 e	40.0	60.0	61.1	13.6	47.5	10.8	31.6	17.1	72.2	10.1	59.4	0.1	0.2	26.2	73.5
	PS-008 Avg.	**24.3**	**75.7**	**60.5**	**11.7**	**48.8**	**7.4**	**32.8**	**14.6**	**84.7**	**10.0**	**74.1**	**0.1**	**0.4**	**11.1**	**88.4**
	Patients' Avg.	**43.8**	**56.2**	**46.8**	**16.6**	**30.2**	**19.8**	**40.4**	**38.1**	**73.6**	**26.1**	**47.4**	**1.6**	**8.7**	**10.1**	**79.6**
	SD	**26.6**	**26.6**	**16.9**	**14.5**	**16.7**	**8.7**	**26.3**	**25.1**	**18.0**	**24.5**	**24.5**	**1.2**	**7.4**	**4.2**	**9.0**

TIL anti-tumor-activity was assessed. The ability to grow TIL, as well as autologous primary prostate cancer cultures from seven patients (PS 002 to 008), enabled us to test TIL for specific tumor recognition. In three patients (PS-003, PS-004, and PS-008) we were able to grow two autologous primary prostate cancer cultures and TIL were tested against both targets. TIL and tumor target cells were co-cultured for 18 h in 96-well plates, supernatants collected and IFNγ levels determined by ELISA. Most clinical ACT protocols demand a minimum secretion of 200 pg/ml IFNγ and at least twice as much as background (TIL only or co-culture of TIL with HLA-mismatched primary PCa cultures of a different patient).[36] TIL derived from five of the seven patients (PS-002, 003, 004, 006 and PS-008) demonstrated specific IFNγ secretion when co-cultured with autologous tumor compared with TIL only (Table 6, Figure 4a). We could observe that different TIL cultures of the same patient had different anti-tumor reactivity, e.g. one TIL culture of patients PS-002, PS-003 and PS-008 met the IFNγ criteria and the other one did not (Table 6). IFNγ secretion of TIL PS-008a was evaluated pre and post REP (Figure 4b). Although TIL PS-008a secreted more IFNγ pre-REP (791 pg/ml) compared with post-REP (281 pg/ml), also the background ("TIL only") was higher pre REP (442 pg/ml) compared with post-REP (131 pg/ml). Adding MHC-class I blocking antibody to the co-culture of post-REP TIL PS-008a reduced the IFNγ secretion to background level (136 pg/ml) (Figure 4b).

Noteworthy, in five out of seven (71%) patients we could generate at least one TIL culture that demonstrated specific anti-tumor reactivity after expansion, making these patients theoretically eligible for TIL ACT (Table 6).

Anti-tumor reactivity was further confirmed by induced expression of the co-stimulatory receptors CD137 (4-1BB) and CD134 (OX40), as well as CD107a, known to be upregulated upon specific T cell activation (Figure 4c). 4-1BB and OX40 expression were upregulated following co-culture compared with "TIL only"

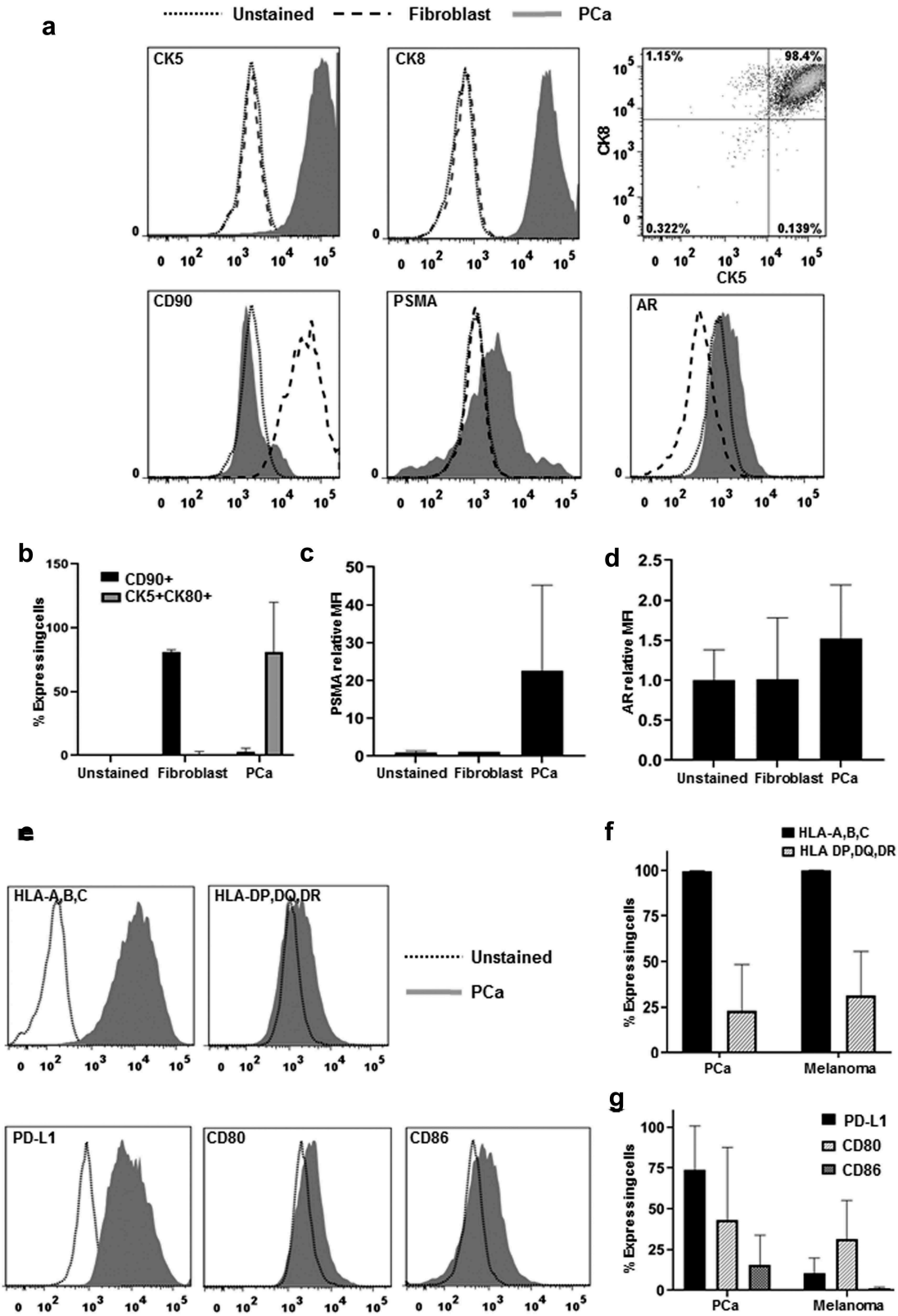

Figure 2. Characterization of primary prostate cancer cultures (PCa). (A) Representative FACS graphs (PS-006) of epithelial markers cytokeratin 5 (CK5) and 8 (CK8), prostate-specific membrane antigen (PSMA), androgen receptor (AR) and the fibroblast surface marker CD90. Fibroblast cells and unstained sample of PS-006 served as controls. (B–D): Average expression of CK5, CK8, and CD90 (B), PSMA (C) and AR (D) in six patients (PS-002-006 and PS-008). (E) Representative FACS histogram plots (PS-008) of HLA-ABC, HLA-DP, DQ, DR, PD-L1, CD80 and CD86. Unstained samples served as control. (F,G): Average expression of HLA class 1 and class 2 (F) and PD-L1, CD80 and CD86 (G) in PCa cultures (n = 3) and melanoma cultures (n = 4).

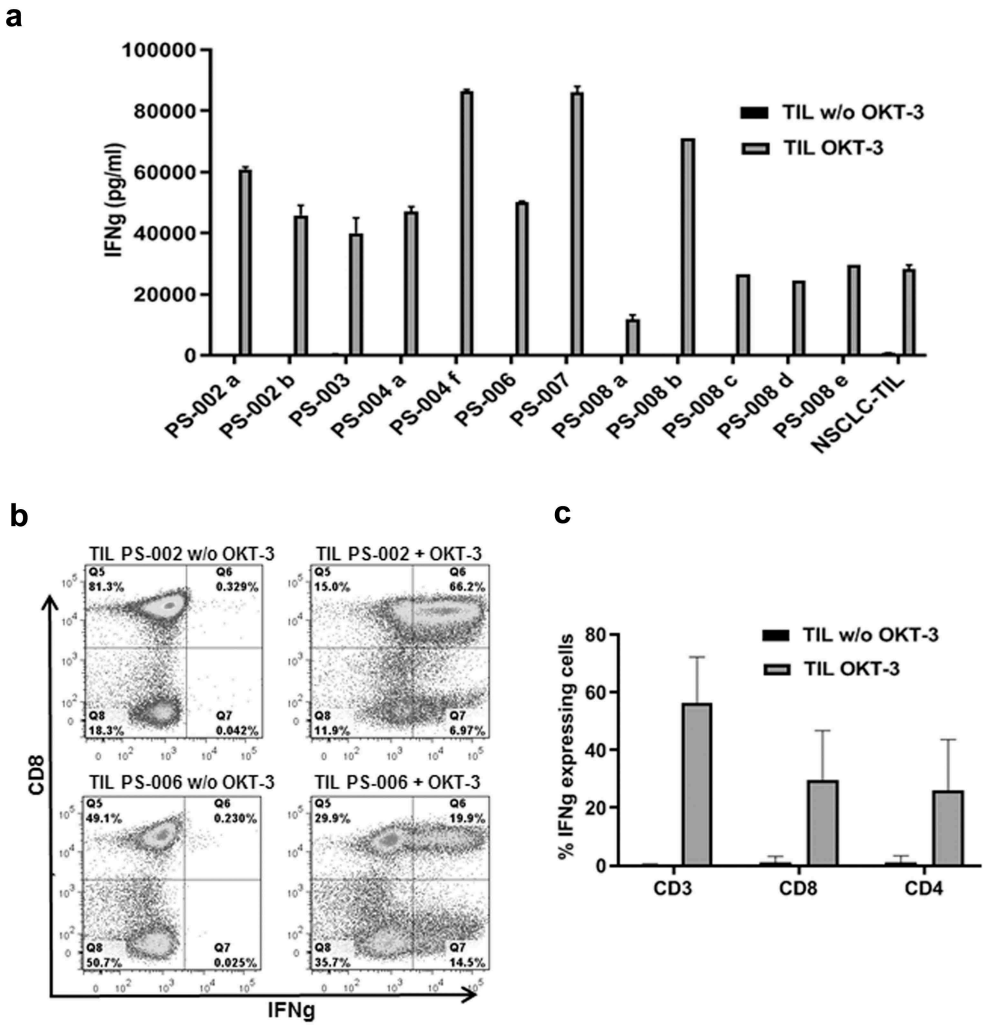

Figure 3. Prostate TIL functionality. (A) 12 TIL cultures from six PCa patients were stimulated overnight with the anti-CD3 antibody OKT-3 (10 µg/ml) and IFNγ secretion (pg/ml) was measured by ELISA and compared to non-stimulated TIL. (B–C) Frequency of IFNγ producing prostate-TIL reactivity measured by intracellular IFNγ flow cytometry after OKT-3 stimulation. (B) Representative FACS plots (PS-002 and PS-006), (C) Frequency of IFNγ producing cells within CD3, CD8 and CD4 T cells subsets (n = 11).

Table 6. IFN-γ secretion (pg/ml) after co-culture of TIL with autologous PCa tumors. Individual TIL cultures from the same patients are named "a" or "b" and different PCa from the same patient target 1 or 2. Values in bold indicate specific anti-tumor reactivity, defined as IFNγ secretion of at least 200 pg/ml and at least twice the value of TIL only. Co-cultures were performed at an E:T ratio of 4:1 (PS-002, 006, 008) or 10:1 (PS-003, 004, 005, 007) 10:1. *N/A, not available*

TIL	TIL only	TIL + target 1	TIL + target 2	At least one reactive TIL
PS-002 a	69	**479**	*N/A*	**Yes**
PS-002 b	<16	<16	*N/A*	
PS-003 a	267	**1677**	**4547**	**Yes**
PS-003 b	356	324	*N/A*	
PS-004 a	184	**585**	**743**	**Yes**
PS-004 b	98	**587**	**232**	
PS-005	<16	<16	*N/A*	**No**
PS-006	361	651	**772**	**Yes**
PS-007 a	<16	<16	*N/A*	**No**
PS-007 b	<16	<16	*N/A*	
PS-008 a	131	**615**	**281**	**Yes**
PS-008 b	96	156	28	

on CD8 (41BB, 9 ± 6% *vs* 3 ± 2%; OX40, 9 ± 6% *vs* 4 ± 3%, n = 7) as well as on CD4 T cells (4-1BB, 12 ± 13% *vs* 5 ± 6%; OX40, 28 ± 11% *vs* 14 ± 10%; n = 7) (Figure 4d).

Chemokine profiling of prostate-TIL

In order to mediate an effective immune response trafficking of TIL to the tumor site is of high importance. Therefore, we

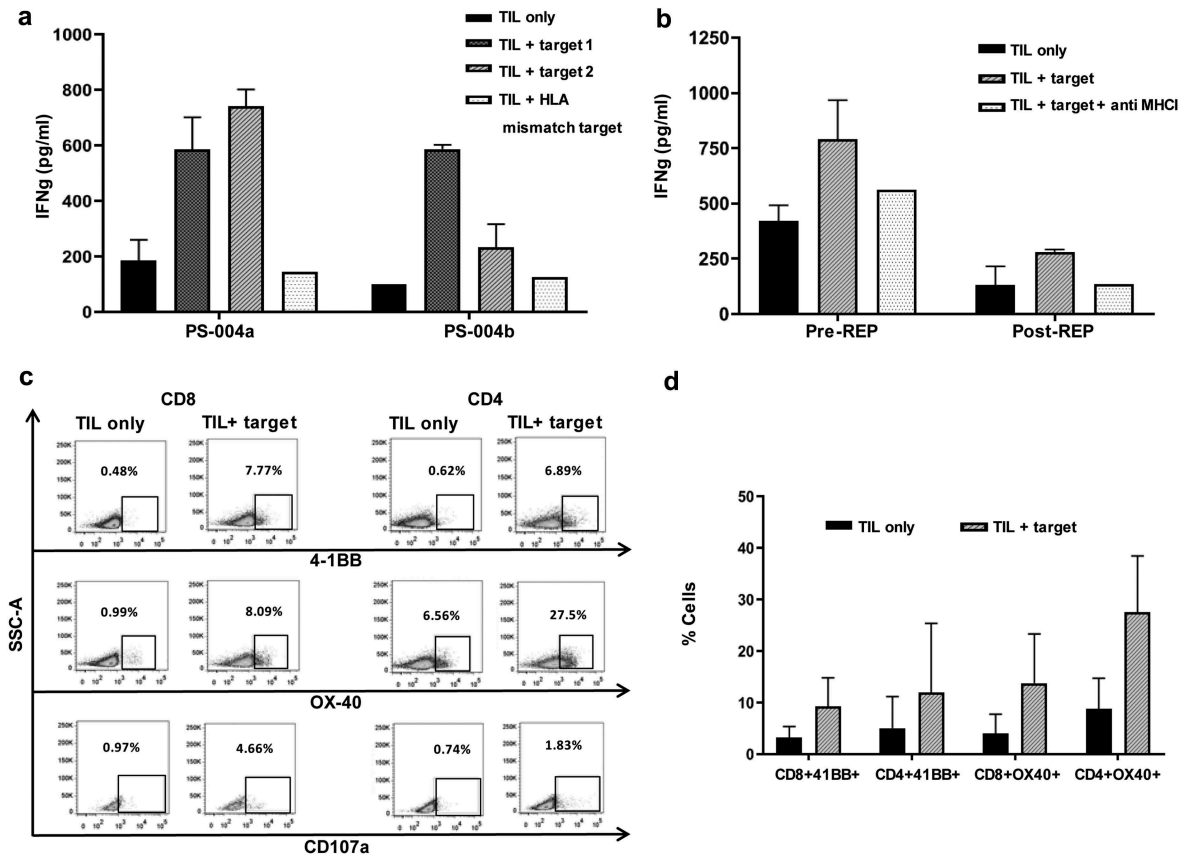

Figure 4. Anti-tumor reactivity of post-REP prostate TIL. Anti-tumor reactivity measured after co-culture with PCa cultures as target. (A) Two TIL cultures of patient PS-004 were co-cultured with two different autologous PCa cultures (Target 1 and 2), HLA-mismatched PCa cultures from PS-006 ("HLA-mismatched target") or without exposure to tumor cells (TIL only). IFNγ secretion (pg/ml) was measured by ELISA. Error bars represent the standard deviation of triplicate repetitions. (B) IFNγ secretion (pg/ml) after co-culture of pre-REP and post-REP TIL of PS-008 with autologous PCa with or without the addition of anti-MHC class 1 blocking antibody. (C) Representative FACS analysis of 4-1BB, OX-40 and CD107a for TIL only and after co-culture with autologous PCa target cells. (D) Average expression of 4-1BB and OX40 on CD8 or CD4 after co-culture (n = 7).

analyzed the expression of six chemokine receptors (CCR2, CCR4, CCR5, CCR7, CXCR2, CXCR3) on 15 post-REP TIL cultures derived from all eight patients. Figure 5b demonstrates high expression of CCR2 and CXCR3, moderate expression of CCR4 and CCR5 and low expression of CCR7 and CXCR2. The chemokine receptors were expressed on CD8 as well as on CD4 cells (Figure 5c). We further compared the chemokine receptor profile of two post-REP TIL to their pre-REP TIL cultures and found a similar distribution of the receptors, with the exception of CCR5 which was low on pre-REP TIL (Figure 5d).

TIL isolated from TRUS-Bx

Next, we tested whether TIL could even be isolated from transrectal ultrasound–guided prostate biopsies (TRUS-Bx). The TRUS-biopsies were approximately 1.3 mm in diameter and 12 mm in length, even smaller than the punch biopsies. Two TRUS-biopsies each were obtained from seven patients (Pbx-001-007). The two biopsies were cut into 4–12 small fragments, 0.5–1 mm,[3] and plated, as described before, with complete media and IL-2 in 24-well plates. Despite the extremely small tissue size, TIL outgrowth was observed in two of the seven patients (Pbx-001 and Pbx-006). Pbx-001 reached a total cell number of 2.6x10e6 on day 40 and Pbx-006 a total cell number of 8x10e4 on day 27. Cells were collected, further expanded in a small-scale rapid expansion procedure and achieved a fold expansion of 523-fold and 760-fold, respectively **(Supplementary Fig. S7A** and **S7B)**. The average frequency of CD3 T cells was 95 ± 2%, including 52 ± 11% CD8 T cells and 48 ± 11% CD4 T cells. The average percentage of PD1 on CD3 T cells was 38 ± 5% and for CD28 70 ± 30%. FACS analysis performed on post-REP TRUS-biopsies derived-TIL showed similar results compared with punch-biopsy derived-TIL **(Supplementary Fig. S7C-E)**. Also, TRUS-biopsies derived-TIL demonstrated functionality, assessed by OKT-3 stimulation followed by measurement of IFNγ secretion by ELISA **(Supplementary Fig. S7F)**.

Discussion

Advanced PCa patients have a poor prognosis and lack curable treatment options, underlining the need to design novel therapeutic strategies. The main objective of the current study was to examine the feasibility of generating TIL cultures from patients with prostate cancer and assess their functionality and anti-tumor reactivity. In the last years, several studies demonstrated the successful establishment of TIL cultures from multiple tumor types including melanoma, lung, breast, pancreatic, renal and bladder cancer,[9,15,36-41] but not prostate cancer, maybe because it

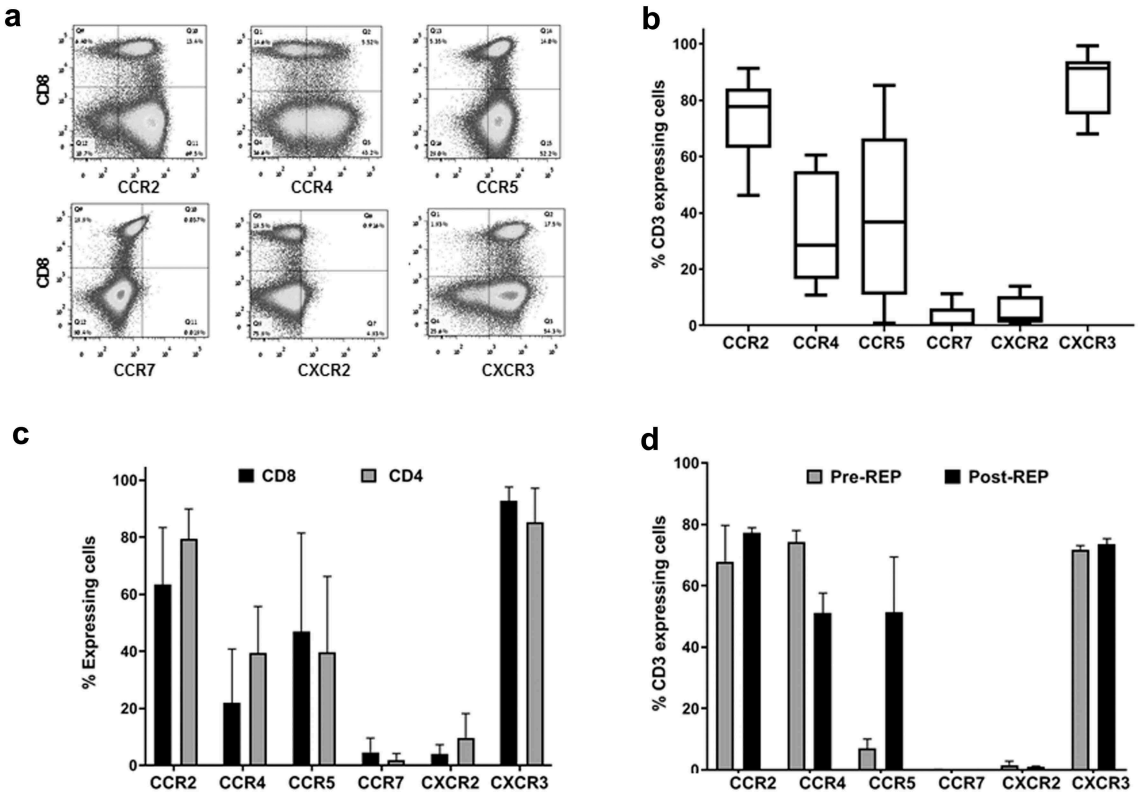

Figure 5. Chemokine profiling of prostate-TIL. (A) Representative dot plots of PS-008. Average expression of chemokine receptors on CD3 positive post-REP TIL (B) and CD4 or CD8 T cells (n = 15) (C). Comparison of two post-REP and pre-REP TIL of PS-007 (D).

is often considered to be a cold tumor, with only little T cell infiltration.[7,8,34] In addition, previous works described the prostate microenvironment as immunosuppressive, infiltrated by suppressor cells, such as regulatory T cells and myeloid-derived suppressor cells.[16,17] Also, stromal cells may contribute to T cells dysfunction, as evidenced in a pancreatic cancer model.[42]

Only a few studies have reported the presence of TIL in the prostate gland.[16,21-24] However, to the best of our knowledge, TIL were never isolated and expanded under conditions suitable for adoptive cell therapy.

To test if TIL can be established from prostate specimens under GMP conditions, we obtained tumor tissue samples from prostatic cancer patients with high tumor burden. Since this study describes the pre-clinical development of TIL ACT, only PCa patients undergoing resection for clinical indications could be included in this study. Primary tumor tissues were therefore obtained by punch biopsies following radical prostatectomy or transrectal ultrasound–guided prostate biopsies.

Despite the extremely small biopsy size of only 0.03 cm^3 per punch, we were able to isolate TIL from 55% (111 of 201) initiated tumor fragments. 1.3x10e5 – 7.2x10e6 TIL were obtained from each patient.

To be of clinical relevance, larger amount of starting material is an indispensable parameter. In comparison, the average biopsy size obtained from 105 metastatic melanoma patients, previously treated with TIL ACT at our center, was 24 $cm.^3$ Despite this constraint, we were able to isolate TIL for all eight PCa patients, showing the potential of this tumor type to be a candidate for TIL therapy with the appropriate amount of tissue.

TIL from all patients were successfully expanded in a 2-weeks rapid expansion procedure with an average fold expansion of 475, demonstrating the proliferative potential of prostate-TIL. Expanded TIL were functional, demonstrated by their high release of IFNγ upon nonspecific (OKT-3) stimulation. The average frequency of expanded TIL cultures showed a ratio of 43% CD8 to 57% CD4 T cells. Prostate-TIL revealed in general a similar phenotype in comparison with melanoma-TIL and NSCLC-TIL, regarding CD8:CD4 ratio and the expression of PD-1. CD28 expression was also similar to melanoma-TIL, but significantly higher than levels measured in NSCLC-TIL. Prostate-TIL were mostly (80%) effector memory T cells (TEM), while melanoma-TIL revealed an almost exclusive TEM phenotype (97%).

Following the establishment of low passage prostate cell lines, the anti-tumor reactivity of TIL was examined. TIL from five out of seven patients demonstrated anti-tumor reactivity against autologous tumors, measured by IFNγ secretion and upregulation of the co-stimulatory molecules CD137 and CD134. These results demonstrate the existence of anti-tumor reactive TIL recognizing autologous prostate tumor cells. Importantly, TIL maintained their reactivity after ex vivo expansion, which is a critical parameter for a potential ACT treatment.

In order to achieve a successful anti-tumor immune response migration and infiltration of T cells following adoptive transfer to the tumor tissue is a critical step. The interaction between chemokine receptors on T cells and tumor-secreted chemokines has been shown to influence trafficking of T cells into the tumor microenvironment.[43,44] Our analysis showed that several

chemokine receptors were abundantly expressed on post-REP TIL including CCR5 and CXCR3, recently reported to correlate with better outcome of ACT therapy.[45]

The ability to grow TIL along with autologous tumor cultures provides a platform to investigate the existence of TIL against tumor-specific mutation, as a potential strategy for the future development of a personalized based vaccine or ACT with T cells targeting mutation-derived neo-antigens. In addition, this platform may be useful to identify T cells with reactivity against prostate cancer-associated antigens, such as PSA.

In conclusion: Previous studies described the presence of nonfunctional TIL in PCa patients and the inability to stimulate them.[6,24,26] However, our data demonstrates that isolated prostate-TIL can be expanded and re-activated. These TIL were functional and anti-tumor specific. This data provides the rational to develop TIL ACT for prostate cancer patients.

Disclosure of potential conflicts of interest

No potential conflicts of interest were disclosed.

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References

1. Venturini NJ, Drake CG. Immunotherapy for Prostate Cancer. Cold Spring Harb Perspect Med. 20182019;9(5). doi:10.1101/cshperspect.a030627.
2. Heidenreich A, Bellmunt J, Bolla M, Joniau S, Mason M, Matveev V, Mottet N, Schmid H-P, van der Kwast T, Wiegel T, et al. EAU guidelines on prostate cancer. Part 1: screening, diagnosis, and treatment of clinically localised disease. Eur Urol. 2011;59:61–71. doi:10.1016/j.eururo.2010.10.039.
3. Silvestri I, Cattarino S, Giantulli S, Nazzari C, Collalti G, Sciarra A. A Perspective of Immunotherapy for Prostate Cancer. Cancers. 2016;8. doi:10.3390/cancers8070064.
4. Maia MC, Hansen AR. A comprehensive review of immunotherapies in prostate cancer. Crit Rev Oncol Hematol. 2017;113:292–303. doi:10.1016/j.critrevonc.2017.02.026.
5. Karantanos T, Corn PG, Thompson TC. Prostate cancer progression after androgen deprivation therapy: mechanisms of castrate resistance and novel therapeutic approaches. Oncogene. 2013;32:5501–5511. doi:10.1038/onc.2013.206.
6. Hammerstrom AE, Cauley DH, Atkinson BJ, Sharma P. Cancer immunotherapy: sipuleucel-T and beyond. Pharmacother. 2011;31:813–828. doi:10.1592/phco.31.8.813.
7. Goswami S, Aparicio A, Subudhi SK. Immune Checkpoint Therapies in Prostate Cancer. Cancer J. 2016;22:117–120. doi:10.1097/PPO.0000000000000176.
8. De Velasco MA, Uemura H. Prostate cancer immunotherapy: where are we and where are we going? Curr Opin Urol. 2018;28:15–24. doi:10.1097/MOU.0000000000000462.
9. Andersen R, Donia M, Ellebaek E, Borch TH, Kongsted P, Iversen TZ, Hölmich LR, Hendel HW, Met Ö, Andersen MH, et al. Long-lasting complete responses in patients with metastatic melanoma after adoptive cell therapy with Tumor-Infiltrating Lymphocytes and an attenuated IL2 regimen. Clin Cancer Res. 2016;22:3734–3745. doi:10.1158/1078-0432.CCR-15-1879.
10. Besser MJ, Shapira-Frommer R, Itzhaki O, Treves AJ, Zippel DB, Levy D, Kubi A, Shoshani N, Zikich D, Ohayon Y, et al. Adoptive transfer of tumor-infiltrating lymphocytes in patients with metastatic melanoma: intent-to-treat analysis and efficacy after failure to prior immunotherapies. Clin Cancer Res. 2013;19:4792–4800. doi:10.1158/1078-0432.CCR-13-0380.
11. Dudley ME, Gross CA, Somerville RP, Hong Y, Schaub NP, Rosati SF, White DE, Nathan D, Restifo NP, Steinberg SM, et al. Randomized selection design trial evaluating CD8+--enriched versus unselected tumor-infiltrating lymphocytes for adoptive cell therapy for patients with melanoma. J Clin Oncol. 2013;31:2152–2159. doi:10.1200/JCO.2012.46.6441.
12. Goff SL, Dudley ME, Citrin DE, Somerville RP, Wunderlich JR, Danforth DN, Zlott DA, Yang JC, Sherry RM, Kammula US, et al. Randomized, prospective evaluation comparing intensity of lymphodepletion before adoptive transfer of Tumor-Infiltrating Lymphocytes for patients with metastatic melanoma. J Clin Oncol. 2016;34:2389–2397. doi:10.1200/JCO.2016.66.7220.
13. Pilon-Thomas S, Kuhn L, Ellwanger S, Janssen W, Royster E, Marzban S, Kudchadkar R, Zager J, Gibney G, Sondak VK, et al. Efficacy of adoptive cell transfer of tumor-infiltrating lymphocytes after lymphopenia induction for metastatic melanoma. J Immunother. 2012;35:615–620. doi:10.1097/CJI.0b013e31826e8f5f.
14. Radvanyi LG, Bernatchez C, Zhang M, Fox PS, Miller P, Chacon J, Wu R, Lizee G, Mahoney S, Alvarado G, et al. Specific lymphocyte subsets predict response to adoptive cell therapy using expanded autologous tumor-infiltrating lymphocytes in metastatic melanoma patients. Clin Cancer Res. 2012;18:6758–6770. doi:10.1158/1078-0432.CCR-12-1177.
15. Ben-Avi R, Farhi R, Ben-Nun A, Gorodner M, Greenberg E, Markel G, Schachter J, Itzhaki O, Besser MJ. Establishment of adoptive cell therapy with tumor infiltrating lymphocytes for non-small cell lung cancer patients. Cancer Immunol Immunother. 2018;67:1221–1230. doi:10.1007/s00262-018-2174-4.
16. Strasner A, Karin M. Immune infiltration and prostate cancer. Front Oncol. 2015;5:128. doi:10.3389/fonc.2015.00128.
17. Bryant G, Wang L, Mulholland DJ. Overcoming oncogenic mediated tumor immunity in prostate cancer. Int J Mol Sci. 2017;18. doi:10.3390/ijms18071542.
18. Chalmers ZR, Connelly CF, Fabrizio D, Gay L, Ali SM, Ennis R, Schrock A, Campbell B, Shlien A, Chmielecki J, et al. Analysis of 100,000 human cancer genomes reveals the landscape of tumor mutational burden. Genome Med. 2017;9:34. doi:10.1186/s13073-017-0424-2.
19. Berger MF, Lawrence MS, Demichelis F, Drier Y, Cibulskis K, Sivachenko AY, Sboner A, Esgueva R, Pflueger D, Sougnez C, et al. The genomic complexity of primary human prostate cancer. Nature. 2011;470:214–220. doi:10.1038/nature09744.
20. Yarchoan M, Johnson BA 3rd, Lutz ER, Laheru DA, Jaffee EM. Targeting neoantigens to augment antitumour immunity. Nat Rev Cancer. 2017;17:209–222. doi:10.1038/nrc.2016.154.
21. Solinas C, Chanza NM, Awada A, Scartozzi M. The immune infiltrate in prostate, bladder and testicular tumors: an old friend for new challenges. Cancer Treat Rev. 2017;53:138–145. doi:10.1016/j.ctrv.2016.12.004.
22. Ness N, Andersen S, Valkov A, Nordby Y, Donnem T, Al-Saad S, Busund L-T, Bremnes RM, Richardsen E. Infiltration of CD8+ lymphocytes is an independent prognostic factor of biochemical failure-free survival in prostate cancer. Prostate. 2014;74:1452–1461. doi:10.1002/pros.22862.
23. Flammiger A, Bayer F, Cirugeda-Kuhnert A, Huland H, Tennstedt P, Simon R, Minner S, Bokemeyer C, Sauter G, Schlomm T, et al. Intratumoral T but not B lymphocytes are related to clinical outcome in prostate cancer. Acta Pathol Microbiol Immunol Scand. 2012;120:901–908. doi:10.1111/j.1600-0463.2012.02924.x.
24. Ebelt K, Babaryka G, Frankenberger B, Stief CG, Eisenmenger W, Kirchner T, Schendel DJ, Noessner E. Prostate cancer lesions are surrounded by FOXP3+, PD-1+ and B7-H1+ lymphocyte clusters. Eur J Cancer. 2009;45:1664–1672. doi:10.1016/j.ejca.2009.02.015.
25. Bronte V, Kasic T, Gri G, Gallana K, Borsellino G, Marigo I, Battistini L, Iafrate M, Prayer-Galetti T, Pagano F, et al. Boosting antitumor responses of T lymphocytes infiltrating human prostate cancers. J Exp Med. 2005;201:1257–1268. doi:10.1084/jem.20042028.

26. Ebelt K, Babaryka G, Figel AM, Pohla H, Buchner A, Stief CG, Eisenmenger W, Kirchner T, Schendel DJ, Noessner E. Dominance of CD4+ lymphocytic infiltrates with disturbed effector cell characteristics in the tumor microenvironment of prostate carcinoma. Prostate. 2008;68:1–10. doi:10.1002/pros.20661.
27. Wheeler TM, Lebovitz RM. Fresh tissue harvest for research from prostatectomy specimens. Prostate. 1994;25:274–279. doi:10.1002/pros.2990250507.
28. Silen A, Wiklund B, Norlen BJ, Nilsson S. Evaluation of a new tumor marker for cytokeratin 8 and 18 fragments in healthy individuals and prostate cancer patients. Prostate. 1994;24:326–332. doi:10.1002/pros.2990240609.
29. Itzhaki O, Hovav E, Ziporen Y, Levy D, Kubi A, Zikich D, Hershkovitz L, Treves AJ, Shalmon B, Zippel D, et al. Establishment and large-scale expansion of minimally cultured "young" tumor infiltrating lymphocytes for adoptive transfer therapy. J Immunother. 2011;34:212–220. doi:10.1097/CJI.0b013e318209c94c.
30. van Dongen JJ, Langerak AW, Bruggemann M, Evans PA, Hummel M, Lavender FL, Delabesse E, Davi F, Schuuring E, García-Sanz R, et al. Design and standardization of PCR primers and protocols for detection of clonal immunoglobulin and T-cell receptor gene recombinations in suspect lymphoproliferations: report of the BIOMED-2 Concerted Action BMH4-CT98-3936. Leukemia. 2003;17:2257–2317. doi:10.1038/sj.leu.2403202.
31. Wang S, Huang S, Zhao X, Zhang Q, Wu M, Sun F, Han G, Wu D. Enrichment of prostate cancer stem cells from primary prostate cancer cultures of biopsy samples. Int J Clin Exp Pathol. 2014;7:184–193.
32. Buhler P, Wolf P, Katzenwadel A, Schultze-Seemann W, Wetterauer U, Freudenberg N, Elsässer-Beile U. Primary prostate cancer cultures are models for androgen-independent transit amplifying cells. Oncol Rep. 2010;23:465–470.
33. Peehl DM. Primary cell cultures as models of prostate cancer development. Endocr Relat Cancer. 2005;12:19–47. doi:10.1677/erc.1.00795.
34. Abrahams NA, Ormsby AH, Brainard J. Validation of cytokeratin 5/6 as an effective substitute for keratin 903 in the differentiation of benign from malignant glands in prostate needle biopsies. Histopathol. 2002;41:35–41. doi:10.1046/j.1365-2559.2002.01425.x.
35. Caspar A, Mostertz J, Leymann M, Ziegler P, Evert K, Evert M, Zimmermann U, Brandenburg L-O, Burchardt M, Stope MB. In vitro cultivation of primary prostate cancer cells alters the molecular biomarker pattern. In Vivo. 2016;30:573–579.
36. Besser MJ, Treves AJ, Itzhaki O, Hardan I, Nagler A, Papa MZ, Catane R, Winkler E, Shalmon-Zifroni B, Schachter J. Adoptive cell therapy for metastatic melanoma patients: pre-clinical development at the sheba medical center. Isr Med Assoc J. 2006;8:164–168.
37. Baldan V, Griffiths R, Hawkins RE, Gilham DE. Efficient and reproducible generation of tumour-infiltrating lymphocytes for renal cell carcinoma. Br J Cancer. 2015;112:1510–1518. doi:10.1038/bjc.2015.96.
38. Poch M, Hall M, Joerger A, Kodumudi K, Beatty M, Innamarato PP, Hoadley KA, Print C, Knowlton N, Black MA, et al. Expansion of tumor infiltrating lymphocytes (TIL) from bladder cancer. Oncoimmunology. 2018;7:e1476816. doi:10.1080/2162402X.2018.1490854.
39. Hall M, Liu H, Malafa M, Centeno B, Hodul PJ, Pimiento J, Pilon-Thomas S, Sarnaik AA. Expansion of tumor-infiltrating lymphocytes (TIL) from human pancreatic tumors. J Immunother Cancer. 2016;4:61. doi:10.1186/s40425-016-0164-7.
40. Lee HJ, Kim YA, Sim CK, Heo SH, Song IH, Park HS, Park SY, Bang WS, Park IA, Lee M, et al. Expansion of tumor-infiltrating lymphocytes and their potential for application as adoptive cell transfer therapy in human breast cancer. Oncotarget. 2017;8:113345–113359. doi:10.18632/oncotarget.23007.
41. Turcotte S, Gros A, Hogan K, Tran E, Hinrichs CS, Wunderlich JR, Dudley ME, Rosenberg SA. Phenotype and function of T cells infiltrating visceral metastases from gastrointestinal cancers and melanoma: implications for adoptive cell transfer therapy. J Immunol. 2013;191:2217–2225. doi:10.4049/jimmunol.1300538.
42. Feig C, Jones JO, Kraman M, Wells RJ, Deonarine A, Chan DS, Connell CM, Roberts EW, Zhao Q, Caballero OL, et al. Targeting CXCL12 from FAP-expressing carcinoma-associated fibroblasts synergizes with anti-PD-L1 immunotherapy in pancreatic cancer. Proc Natl Acad Sci U S A. 2013;110:20212–20217. doi:10.1073/pnas.1320318110.
43. Balkwill F. Cancer and the chemokine network. Nat Rev Cancer. 2004;4:540–550. doi:10.1038/nrc1388.
44. Viola A, Molon B, Contento RL. Chemokines: coded messages for T-cell missions. Front Biosci. 2008;13:6341–6353. doi:10.2741/3158.
45. Bedognetti D, Spivey TL, Zhao Y, Uccellini L, Tomei S, Dudley ME, Ascierto ML, De Giorgi V, Liu Q, Delogu LG, et al. CXCR3/CCR5 pathways in metastatic melanoma patients treated with adoptive therapy and interleukin-2. Br J Cancer. 2013;109:2412–2423. doi:10.1038/bjc.2013.557.