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Immune responses against autologous tumor and human papilloma virus in lymph nodes from patients with penile cancer

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Purpose: Nearly half of penile cancers are related to human papillomavirus (HPV) infection. Investigations of tumor- and HPV-specific T cell reactivity in regional lymph nodes (LNs) from patients with penile cancer are warranted.

Materials and Methods: In this study, single-cell suspensions from LNs and peripheral blood from 11 patients with penile cancer were stained with antibodies for lymphocyte markers and analyzed by fluorescence-activated cell sorting (FACS). DNA was extracted from the tumor tissue and HPV status was investigated by PCR.

Results: T cell reactivity against autologous tumor extract and against the HPV vaccine Gardasil was tested by flow-cytometric assay of specific cell-mediated immune response in activated whole blood (FASCIA). $CD4^+/CD8^+$ ratios were significantly lower in HPV-positive LNs ($p<0.05$). Immune responses to tumor extract assessed by blast transformation and expansion *in vitro,* of either $CD4^+$ or $CD8^+$ T cells, were found in 9 of 13 LNs (69%). Thus, 5 of 6 tested patients demonstrated T cell recognition of tumor-associated antigen(s). In HPV-positive patients, dose-dependent T cell responses against L1 (late) HPV proteins (Gardasil vaccine) were demonstrated.

Conclusions: LN-derived T cells from patients with penile cancer recognize tumor antigen(s) and in HPV-positive cases, there is a response against L1 (late) HPV proteins, being constituents of the Gardasil vaccine.

Keywords: Adoptive immunity; Lymph node excision; Papillomaviridae; Penile neoplasms; T-lymphocytes

INTRODUCTION

Human papillomavirus (HPV) is a cancer-causing DNA virus infecting humans, accounting for 5.2% of cancers worldwide [1]. Penile cancer is rare in developed countries (0.3–1.0 case per 100,000/year) accounting for around 0.4% to 0.6% of all malignancies. Nearly half of all penile cancers are HPV-positive [2]. Among 170 HPV strains [3], HPV16 is the main strain accounting for 68.7% of all penile cancers [2]. The presence of HPV DNA in primary tumors and metastases indicates the potential role of HPV in tumor progression [2,4]. Standard treatment for invasive penile cancer is radical

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Table 1. Patient characteristics and cTNM and pTNM staging of the investigated patients

Patient no.	Age (y)	Circumcised before time of diagnosis, yes/no	Clinical tumor staging	pT-stages	Total number of excised lymph nodes	Total number of metastatic lymph nodes	Treatment after radical surgery
1	84	No	T2N0M0	pT2	4	0	Palliative radiation
2	72	No	T1N0M0	pT1a	3	0	No
3	72	No	T2N0M0	pT2	4	0	No
4	78	No	T1N0M0	pT1a	2	0	No
5	66	No	T1N0M0	pT1	5	0	No
6	50	No	T2N0M0	pT2	4	0	Surgery again 21.5 months later due to metastases
7	80	No	T2N0M0	pT2	7	0	Total amputation of penis 1.5 months later
8	77	No	T1N0M0	pT1	4	0	No
9	78	No	T2N1M0	pT2	8	1	No
10	78	No	T1N0M0	pT1a	4	2	Repeat surgery 2 months later to remove bilateral lymph nodes inguinal and iliac (yield 11/14 metastatic nodes)
11	70	Yes[a]	T2N0M0	pT2	4	0	No

[a]:Patient no. 11 was diagnosed with a phimosis 8 years before radical surgery and then circumcised. One year later, he developed a plasma cell balanitis (Zoon´s balanitis), which was followed. Four years prior to radical surgery, the patient had a noninvasive penile cancer which finally progressed locally.

surgery with or without cisplatin-based chemotherapy [5].

In solid tumors, the histologic status of the regional lymph nodes (LNs) is one of the most important predictors of patient survival [6,7]. The LNs are regarded as the primary compartment where tumor cells and the host immune system interact. The concept of adoptive immunotherapy in cancer is based upon the immunosurveillance hypothesis [8]. There are several indications of HPV-associated malignancies eliciting a virus-specific immune response [9,10]. The presence of tumor-infiltrating lymphocytes (TILs) in cervical cancer correlates with prognosis, and treatment with HPV-TIL infusions demonstrates that durable and complete regression of metastases is immunologically mediated [11]. In adoptive immunotherapy, TILs are a frequently used cell population because antigen-specific T cells are accumulated intratumorally. In addition, tumor-antigen-recognizing lymphocytes occur in tumor-draining LNs in human malignancies such as colon and urinary bladder cancer, where T cells are activated by tumor antigens and presented by professional antigen-presenting cells (APCs) [12,13]. Thus, tumor-specific lymphocytes obtained from LNs may be enriched, undergo *ex vivo* expansion, and be further used for adoptive immunotherapy.

HPV early proteins, E6 and E7, are oncoproteins affecting cell growth control through inactivation of cellular tumor suppressor gene products: p53 and Rb, respectively [14]. E6 and E7 are widely studied in HPV-associated cancer immunotherapy [9-11]. A study comparing the efficiency of HPV-antigen-specific T cells in killing autologous HPV-positive tumor cells in cervical cancer patients suggests that L1-specific $CD8^+$ cytotoxic T lymphocytes are equally as effective as E7-specific $CD8^+$ cytotoxic T lymphocytes [15]. A HPV vaccine study demonstrated that Gardasil® could be highly efficacious against HPV 16/18 [16].

MATERIALS AND METHODS

1. Patients

Eleven patients with invasive penile cancer, staged cT1-2N0-1M0, and aged 50 to 84 years, from Södersjukhuset (Stockholm, Sweden) and Norrlands Universitetssjukhus (Umeå, Sweden), were prospectively enrolled from 2013 to 2015 (Table 1). Oral and written informed consent was obtained from all participants. The study was approved by the regional ethical committee in Umeå University, Umeå, Sweden (approval number: EPN-2013/835-32). All procedures performed in the study conformed to the provisions of the Declaration of Helsinki from 1964 according to the revision in Fortaleza, Brazil, October 2013.

2. Preparation of specimens

One piece of primary tumor was dissected during surgery and used for PCR analyses and as antigen. LNs were identified and excised, and one-half of each LN was used for routine histopathology and immunologic evaluation.

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3. Preparation of single cells

Venous blood, LNs, and tumor specimens were immediately harvested. Peripheral blood mononuclear cells (PBMCs) were purified by Ficoll-Hypaque density gradient centrifugation (Pharmacia, Amersham, Uppsala, Sweden). Single-cell suspensions from tissue specimens were obtained by gentle pressure using a loose-fit glass homogenizer. Cells were washed twice and resuspended in AIM V serum-free medium (Life Technologies, Carlsbad, CA, USA).

4. Flow cytometry (fluorescence-activated cell sorting, FACS)

PBMCs, LNs, and tumor cell suspensions at 0.5x106 cells/sample were washed in PBS containing 2% FCS and 0.05% NaN3 (FACS-buffer). To investigate lymphocyte subtypes and their functions, staining was performed with fluorophore-conjugated antibodies: Blue Live/Dead stain, anti-CD4 Pacific-Blue, anti-CD8 APC, anti-CD19 APC-Cy7, and anti-CD56 PE (Becton Dickinson-BD, Franklin Lakes, NJ, USA). Cells were investigated with LSRFORTESSA (BD) and analyzed by using the FACS DIVA software (BD).

Table 2. Primers used to detect HPV16 and HPV18 infections[a]

Type	Primer	Primer sequence (5′–3′)	Region
MY09	Pr1	CGTCCMARRGGAWACTGATC	L1
MY11	Pr2	GCMCAGGGWCATAAYAATGG	
HPV-16	Pr1	TGCACGCACAAACATATATTATCATG	L1
	Pr2	CCTGTATTGTAATCCTGATACTTTAG	
HPV-18	Pr1	CCAAGTGGCTCTATTGTTACCTC	L1
	Pr2	GAGTGGTATCTACCACAGTAAC	

HPV, human papillomavirus.
[a]:All primers were targeted on the L1 region.

5. Activation of T cells

Tumor samples were homogenized using an Ultra-Turrax homogenizer in 5 volumes (w/v) of RPMI, followed by 5 minutes of denaturation at 98℃. Activation of single-cell suspensions of PBMCs, LN cells, and TILs was tested at 0.5 million cells per tube using tumor homogenate, Gardasil HPV vaccine (0.1–1.0 μg/mL), or pokeweed mitogen (5 mg/mL) (Sigma, St. Louis, MO, USA). Activation was measured by blast transformation (day 7) and cell surface markers by flow-cytometric assay of specific cell-mediated immune response in activated whole blood (FASCIA). The optimal markers for analyzing T helper cells, cytotoxic T cells, B cells, and NK cells, namely, CD4, CD8, CD19, and CD56, were utilized [17].

6. PCR assay

Tumor samples were stored in RNA-later (Ambion, Austin, TX, USA) and extracted for DNA by use of DNAeasy Blood and Tissue kits (Qiagen, Venlo, Netherlands). Primers were used to detect the L1-region of HPV16 and HPV18 (Table 2) [18,19]. HPV18-DNA extracted from HeLa cells and HPV16-DNA (Advanced Biotechnologies, Eldersburg, MD, USA) were used as positive controls. PCR assay was performed by use of a Bio-Rad T100 Thermal Cycler following standard PCR protocol.

7. Statistical analysis

Spearman correlation was used to analyze relations between subpopulations using GraphPad software (GraphPad Software, La Jolla, CA, USA). A p-value <0.05 was considered significant.

Table 3. Laboratory characteristics of lymph node cell populations[a]

Patient no.	Average % $CD4^+$	Average % $CD8^+$	Average % $CD4^+/CD8^+$	Average % $CD19^+$	Average % $CD56^+$	HPV[b]
1	43.90	7.87	6.11	38.00	5.20	ND
2	21.40	4.97	4.46	52.67	0.50	ND
3	49.90	20.00	2.57	41.25	25.40	ND
4	68.40	13.95	4.94	43.35	1.50	ND
5	69.05	5.00	13.81	15.05	0.60	ND
6	59.57	7.37	8.53	21.77	0.83	ND
7	81.30	3.60	19.92	6.67	0.43	No
8	74.00	11.70	5.60	12.40	2.40	Yes
9	38.90	2.55	15.39	33.50	1.25	Yes
10	29.33	39.43	0.75	20.57	12.00	No
11	66.67	5.57	11.97	18.83	0.80	No

HPV, human papillomavirus.
[a]:In samples from patients 7–11, HPV virus was investigated.
[b]:ND denotes not determined, Yes denotes positive detection of HPV virus, and No denotes negative detection of HPV virus.

RESULTS

1. HPV classification of tumors

In 5 of 11 included patients of the prospective cohort, primary tumors were available for DNA analysis. DNA was extracted and investigated using HPV primers for the L1 gene from HPV16 and HPV18. In samples from 2 of 5 (40.0%) tested patients (patients 7–11), we detected HPV virus (Table 3), which was comparable with reported frequencies (46.3%) [20,21].

2. Immunophenotyping of LNs

Single-cell suspensions obtained from tumor, LNs, and peripheral blood were analyzed by FACS (Fig. 1A) [21]. The average fraction of $CD4^+$ T lymphocytes in LNs was between 21% and 81.3% and that of $CD8^+$ T lymphocytes was between 2.5% and 39.4% (Table 3). $CD4^+/CD8^+$ ratios were significantly different between LNs and peripheral blood ($p<0.001$) (Fig. 1B). The $CD4^+/CD8^+$ ratio in PBMCs was 2.5 compared with 10 in LNs (Fig. 1B), demonstrating expansion of $CD4^+$ T lymphocytes in LNs. By comparing lymphocyte populations, we found a significant correlation between $CD8^+$ T lymphocytes and $CD56^+$-expressing lymphocytes (Spearman r=0.67, $p<0.001$) (Fig. 2A). Furthermore, we found an inverse correlation between the fraction of $CD19^+$ B lymphocytes and $CD4^+$ T lymphocytes (Spearman r=-0.71, $p<0.001$) (Fig. 2B). Additionally, we found an inverse correlation between the fraction of $CD19^+$ B lymphocytes and the $CD4^+/CD8^+$ ratio (Spearman r=-0.61, $p<0.001$) (Fig. 2C). When analyzing $CD4^+/CD8^+$ ratios in HPV^+ LNs, we found a significantly decreased $CD4^+/CD8^+$ ratio in LNs derived from HPV-positive patients ($p<0.05$), suggesting a relative increase in virus-recognizing $CD8^+$ T lymphocytes in HPV patients (Fig. 3A).

3. Anti-tumoral reactivity in regional LNs

In 13 available LNs (6 patients), we investigated immune responses toward autologous tumor extract by FASCIA [22]. We observed $CD4^+$ T lymphocyte responses in 6 of 13 LNs (Fig. 3B). Correspondingly, $CD8^+$ T lymphocyte blast responses were seen in 7 of 13 LNs (Fig. 3B). In total, we found an immune response, either $CD4^+$ or $CD8^+$, in 9 of 13 tested LNs. Thus, 5 of 6 (83%) tested patients demonstrated a T cell recognition of autologous tumor antigens.

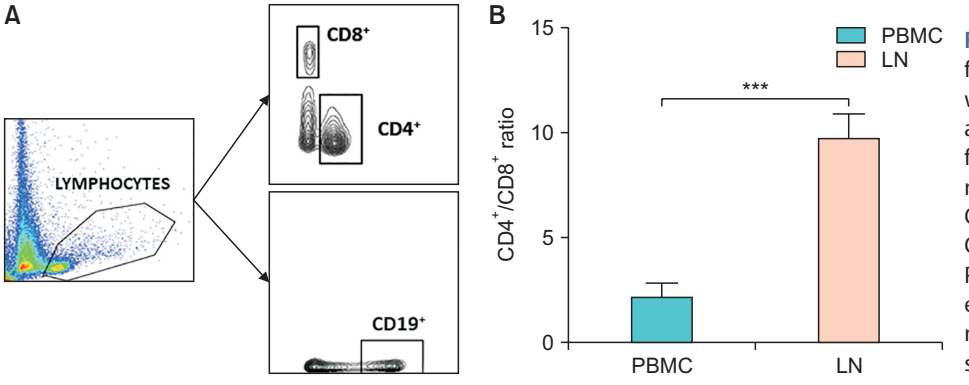

Fig. 1. Single-cell suspensions obtained from tumor, LNs, and peripheral blood were analyzed by FACS (A) and gated according to side and forward scatter followed by lineage-specific cell surface markers for T lymphocytes ($CD4^+$ and $CD8^+$) and B lymphocytes ($CD19^+$). (B) $CD4^+/CD8^+$ ratios between LNs and PBMCs were compared. PBMC, peripheral blood mononuclear cell; LN, lymph node; FACS, fluorescence-activated cell sorting. ***$p<0.001$.

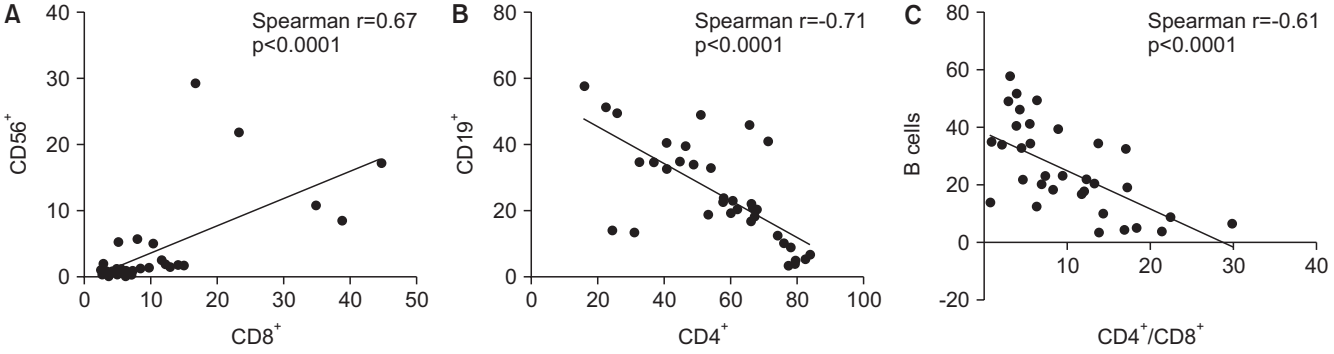

Fig. 2. Single-cell suspensions from lymph nodes were analyzed by FACS (fluorescence-activated cell sorting) by using specific cell surface markers for T lymphocytes ($CD4^+$ and $CD8^+$) and B lymphocytes ($CD19^+$) and correlated with the activation marker $CD56^+$ by using Spearman correlation tests. The correlations between the fraction of (A) $CD8^+$ cytotoxic T cells and $CD56^+$-expressing lymphocytes, (B) $CD19^+$ B lymphocytes and $CD4^+$ T helper cells, and (C) $CD19^+$ B lymphocytes and $CD4^+/CD8^+$ ratios were investigated. Regression coefficients and p-values are given in the figure.

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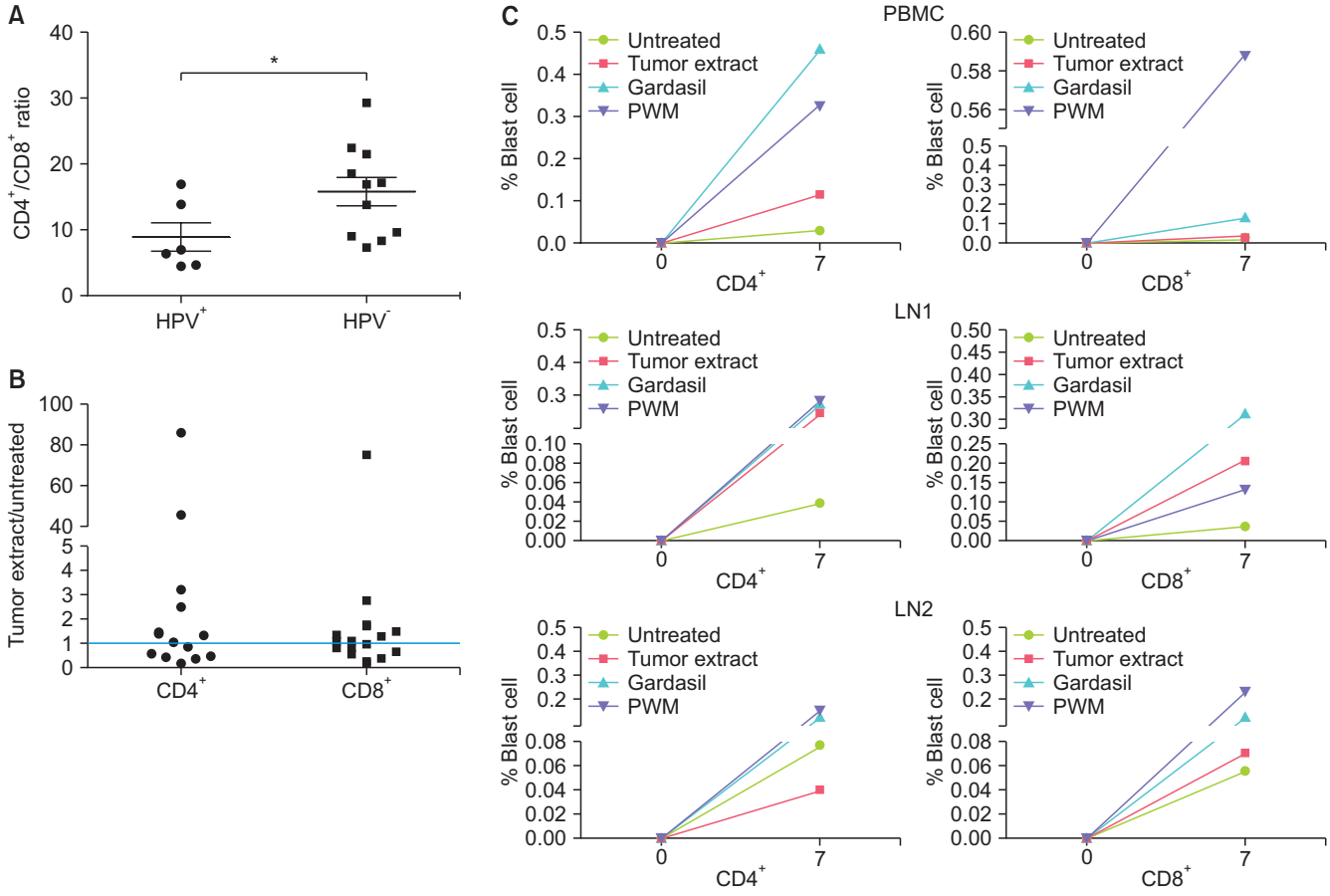

Fig. 3. $CD4^+/CD8^+$ T lymphocyte ratios were compared between HPV-positive LNs and HPV-negative LN (A). Evaluation of immune responses toward autologous tumor extract by FASCIA was carried out in 13 LNs from six patients. The response to tumor extract/untreated was calculated. Responses above 1 (blue line) were considered as tumor-induced T cell responses (B). Lymphocytes were evaluated by stimulation with the Gardasil HPV vaccine containing L1 proteins derived from HPV, and tumor extract from HPV-positive patients, and compared with the general response to PWM and untreated (C). The fraction of blast responding $CD4^+$ and $CD8^+$ T lymphocytes from PBMCs and lymph nodes was evaluated by FASCIA on day 7 from HPV-positive patient no. 8. HPV, human papillomavirus; PBMC, peripheral blood mononuclear cell; LN, lymph node; PWM, pokeweed mitogen; FASCIA, flow-cytometric assay of specific cell-mediated immune response in activated whole blood.

4. HPV-reactivity in regional LNs

Due to the association with HPV infection, we conducted both HPV vaccine and tumor extract-stimulation with LN cells from HPV-positive patients. When using tumor extract as antigen for stimulation, a small T cell response was noted with PBMCs and cells from LNs (Fig. 3C). However, we found strong responses in PBMCs and LN cells in HPV-positive patient no. 8 (Fig. 3C). Both HPV-positive patients (patients 8 and 9) responded to HPV vaccine and to tumor extract. Only absent or very weak responses toward HPV vaccine were noted in HPV-negative patients (not shown), demonstrating that L1 protein responses were restricted to HPV-positive patients. The LN-derived lymphocytes from HPV-positive patient no. 9 responded in a dose-dependent manner against HPV vaccine (Fig. 4A). Investigation of the cell surface expression of the activation marker HLA-DR showed a 10-fold increase from the unstimulated to the HPV virus-stimulated group, substantiating vaccine-dependent T cell activation (Fig. 4B).

DISCUSSION

We have here for the first time demonstrated tumor antigen and HPV-specific T cell responses in LNs from patients with penile cancer. We found that HPV-infected penile cancer patients displayed a significant decrease of the $CD4^+/CD8^+$ ratio in LNs, suggesting activation and expansion of HPV-recognizing $CD8^+$ T lymphocytes (Fig. 3A). Although $CD56^+$ is a known NK cell marker, $CD56^+$ is also expressed on activated cytotoxic $CD8^+$ T lymphocytes [23], suggesting that LNs contain an increased number of activated $CD8^+$ T lymphocytes, further demonstrating their state of activation (Fig. 2A). Additionally, we found activation of $CD4^+$ T_h cells in LNs responding to the HPV-L1 proteins from HPV16 or HPV18 present in the Gardasil HPV vaccine, supporting the concept of HPV-specific immune responses. The FASCIA as-

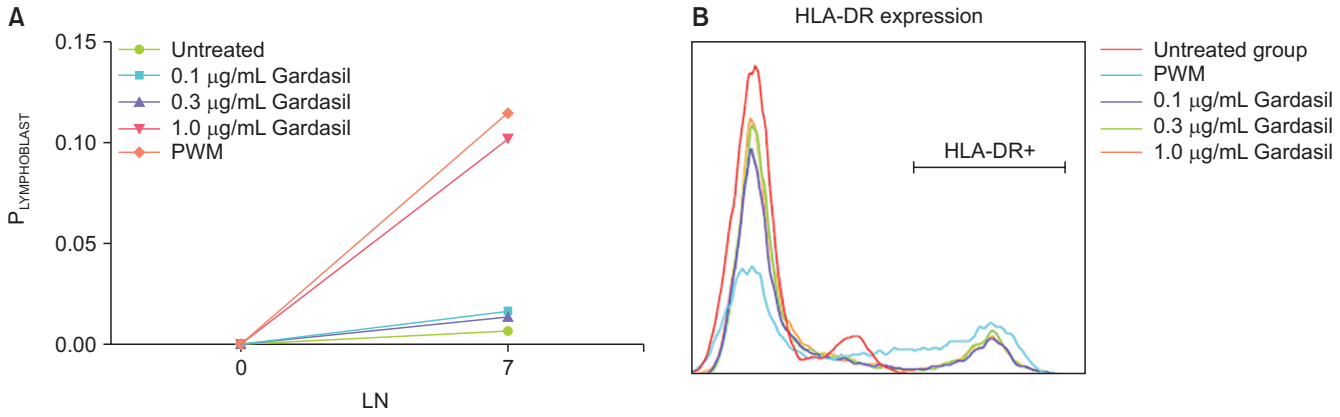

Fig. 4. Lymph node-derived lymphocytes from HPV-positive patient no. 9 were evaluated by FASCIA on day 7 after stimulation with 0.1, 0.3, or 1.0 μg/mL Gardasil HPV vaccine or PWM as a control (A). In the same experiment the cell surface expression of the T cell activation marker HLA-DR was evaluated by FACS (B). PWM, pokeweed mitogen; HPV, human papillomavirus; FASCIA, flow-cytometric assay of specific cell-mediated immune response in activated whole blood; FACS, fluorescence-activated cell sorting.

say used the addition of tumor homogenate or L1 proteins from HPV16 or HPV18 present in the HPV vaccine, and the length of the peptides or the exogenously provided homogenate promotes presentation in the major histocompability complex (MHC) class II pocket and hence stimulation of $CD4^+$ T lymphocytes. The activation of $CD8^+$ T lymphocytes against HPV vaccine was poor in PBMC cultures, but they responded nicely in LNs (Fig. 3C). This suggests cross-presentation in LN cultures where specialized APCs may have the capacity to load MHC class I molecules for $CD8^+$ T cell activation [24].

Interestingly, our data might also confirm the postulated inverse relationship between $CD19^+$ B lymphocytes and the $CD4^+/CD8^+$ T lymphocyte ratio (Fig. 2C). Experimental data from other groups have suggested that regulatory B lymphocytes, Bregs, may suppress the proliferation of helper T1 (Th1) cells. We did not pursue this matter in detail, which would have required a more extensive panel of markers [25,26]. In studies of cervical cancer, HPV infection can affect the host immune responses [27], where accumulation of ineffective $CD8^+$ T lymphocytes was observed in stage II/III CINs [28]. HPV-specific T cell responses seem to be important in cancer defense, since a higher incidence of HPV-related lesions are observed in immunosuppressed individuals after organ transplantation [29]. The increased T cell proliferation in response to Gardasil HPV vaccine compared with tumor extract suggests that HPV vaccine contains higher concentrations of pure antigens compared with autologous tumor extracts. Since we demonstrated a dose-dependent response toward L1-proteins, HPV vaccine may be a source in expansion of HPV-specific T cells in HPV-positive penile cancer patients for adoptive T cell therapy.

To enhance the effect of an adoptive immune response in penile cancer, we suggest using HPV-peptide cocktails promoting $CD8^+$ T cell responses. Compared with autologous tumor extracts, which contain a mixture of unknown proteins, defined HPV peptides might be more efficient. In a recent study using HPV E6 and E7 peptides to stimulate and expand TILs from patients with cervical cancer, two patients experienced complete responses [11]. In addition, HPV-specific $CD8^+$ and $CD4^+$ T cell responses against E6 and E7 proteins were found in patients with cervical cancer [30], suggesting a potential role of specific T cell therapy in HPV malignancies. Thus, the use of E6 and E7 peptides to expand HPV-specific T cells for immunotherapy of patients with penile cancer may be useful. Note that in the most optimal setting, parts of all extracted nodes could have undergone analysis. Yet availability and, in some instances, very small nodes with low volumes of nodal tissue did not allow us to infringe on standard clinical and pathology practices. Furthermore, it would have been more optimal to differentiate between sentinel nodes and nonsentinel nodes, because these different types of LNs hypothetically have different immunologic properties. Although this differentiation was not performed, it is a goal in our forthcoming studies. Note also that two of the patients had three metastatic nodes in total. None of them were analyzed in our study.

CONCLUSIONS

LN-derived T lymphocytes from patients with penile cancer respond to tumor antigen. HPV-positive cases respond to the Gardasil HPV vaccine. In penile cancer, strategies using L1-proteins from HPV as an antigen may be useful for adoptive T cell therapy.

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CONFLICTS OF INTEREST

The authors have nothing to disclose.

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AUTHORS' CONTRIBUTIONS

Research conception and design: Ola Winqvist, Ping-Sheng Hu, Lu Zhang, and Amir Sherif. Data acquisition: Jesper Rosvall, Mats Hedlund, Katrine Riklund, and Amir Sherif. Statistical analysis: Lu Zhang and Ola Winqvist. Data analysis and interpretation: Lu Zhang, Ola Winqvist, A. Ali Zirakzadeh, and Amir Sherif. Drafting of the manuscript: Lu Zhang, Ola Winqvist, and Amir Sherif. Critical revision of the manuscript: Lu Zhang, A. Ali Zirakzadeh, Jesper Rosvall, Mats Hedlund, Ping-Sheng Hu, Katrine Riklund, Amir Sherif, and Ola Winqvist. Obtaining funding: Ola Winqvist, Ping-Sheng Hu, Amir Sherif, and Katrine Riklund. Administrative, technical, or material support: Ola Winqvist, Ping-Sheng Hu, Amir Sherif, and Katrine Riklund. Supervision: Ola Winqvist, Ping-Sheng Hu, and Amir Sherif. Approval of the final manuscript: Lu Zhang, A. Ali Zirakzadeh, Jesper Rosvall, Mats Hedlund, Ping-Sheng Hu, Katrine Riklund, Amir Sherif, and Ola Winqvist.

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