Eur J Dermatol 2016; 26(2): 138-43

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Article accepted on 29/11/2015

Xenogeneic cell-based vaccine therapy for stage III melanoma: safety, immune-mediated responses and survival benefits

Background: New therapies for melanoma have yielded promising results, but their application is limited because of serious side-effects and only moderate impact on patient survival. Vaccine therapies may offer some hope by targeting tumor-specific responses, considering the immunogenic nature of melanomas. *Objectives:* To investigate the safety profile and efficiency of a xenogeneic cell-based vaccine therapy in stage III melanoma patients and evaluate the survival rate in treated patients. *Materials and Methods:* Twenty-seven stage III melanoma patients were immunized with a lyophilized xenogeneic polyantigenic vaccine (XPV) prepared from murine melanoma B16 and carcinoma LLC cells. *Results:* Neither grade III/IV toxicities, nor clinically significant changes in blood and biochemical parameters were noted after an induction course of 10 XPV subcutaneous immunizations. No laboratory or clinical signs of systemic autoimmunity were documented. Following 10 vaccinations, a relative increase in the numbers of circulating memory CD4+CD45RO+ T cells (but not CD8+ CD45RO+ T cells) was observed. Peripheral blood mononuclear cells obtained from XPV-treated patients demonstrated increased proliferative responses to human BRO melanoma-associated antigens and marked increases in serum levels of IFN-γ and IL-8. Serum levels of TNF-α, IL-4 and IL-6 were not affected. The overall five-year survival rate in the treated patients was significantly higher than that in 27 control patients with matched clinical and prognostic characteristics (55% *vs* 18%). *Conclusion:* XPV-based immunotherapy could be maximally effective when started as early as possible before or after surgical excision of the primary tumor and local metastases, i.e. when tumor-mediated suppressive effects on immunity are minimal.

Key words: stage III melanoma, xenogeneic polyantigenic vaccine, toxicity, safety memory T cell, survival rate

The incidence of melanoma is increasing worldwide. Although potentially curable when diagnosed early, advanced melanoma carries a poor prognosis. Chemotherapeutic agents, such as dacarbazine or temozolomideare, are often given to patients for palliative purposes; high-dose interleukin 2 and interferon-α (IFN-α) are immunotherapeutic modalities that could be offered to patients with a good performance status [1]. However, until recently, systemic therapy for advanced melanoma was ineffective. Recent advances in the development of new therapies, such as mitogen-activated protein kinase (MAPK) pathway inhibitors, anti-cytotoxic T-lymphocyte-associated antigen-4 (CTLA-4), and programmed cell death protein 1 (PD-1)/programmed cell death ligand 1 (PD-L1) pathway blocking antibodies, as well as combination strategies of cytotoxic chemotherapy and inhibitors of angiogenesis, have all yielded promising results [2]. It should be stressed that all new therapies have only a moderate impact on patient survival and their application is quite limited due to serious side effects. Thus, the most common side effects of antibodies (Abs) blocking immune checkpoint molecules (CTLA-4 or PD-1) are colitis, hepatitis, dermatitis, hypophysitis, uveitis, diarrhea and pneumonitis [3]. These effects reflect non-selective drug-mediated immunostimulation that triggers a pronounced amplification of inflammatory processes, including those

Abbreviations:

Ab	antibody
IFN	interferon
IL	interleukin
M	mean
PBMC	peripheral blood mononuclear cell
SEM	standard error of the mean
SI	stimulation index
TAA	tumor-associated antigen
TNF	tumor necrosis factor
XPV	xenogeneic polyantigenic vaccine

doi:10.1684/ejd.2016.2733

To cite this article: Seledtsova GV, Shishkov AA, Kaschenko EA, Goncharov AG, Gazatova ND, Seledtsov VI. Xenogeneic cell-based vaccine therapy for stage III melanoma: safety, immune-mediated responses and survival benefits. *Eur J Dermatol* 2016; 26(2): 138-43 doi:10.1684/ejd.2016.2733

of an autoimmune origin. Selective activation of tumor-specific immune responses can be achieved by applying vaccine-based technologies. In fact, melanoma is considered as one of the most immunogenic solid tumors. Hence, there is persistent optimism that vaccine therapies will be able to improve clinical outcomes in melanoma settings in the future. Despite intense research efforts in this field, only a few clinical trials to date have demonstrated significant clinical benefits from melanoma vaccines in high-risk patients [1].

In fact, immunizations with one or several tumor-associated antigenic peptides frequently fail to control the overall tumor development, creating favorable conditions for growth of the tumor cell clones lacking vaccine antigenic determinants [4]. The use of whole tumor cells or lysates derived thereof as vaccines offers several advantages compared to individual TAAs. First, whole tumor cells elicit broad spectrum immune responses to different TAAs. Second, after internalization by antigen-presenting cells (APCs), tumor cell debris facilitate cross-presentation of antigens to CD4+ and CD8+ T cells, thus generating long-term CD8+ T cell memory with CD4+ T cell help [4]. Although both autologous and allogeneic cell vaccines are well studied, we believe in the higher clinical potential of a xenogeneic vaccine approach. On the one hand, TAAs are typically evolutionarily conserved molecules, such that strong homology is observed between human and animal TAAs. On the other hand, small interspecies structural differences in TAAs could be beneficial in terms of cancer vaccine development, as xenogeneic antigens could fit with an 'altered self' paradigm, endowing them with sufficient differences from self-antigens to render them immunogenic. An accumulating body of evidence suggests that xenogeneic vaccines can be very effective in breaking the mechanisms of immune tolerance to low immunogenic human TAAs [4, 5]. A xenogeneic polyantigenic vaccine (XPV) has been developed at our Centre to combine lysed murine B16 melanoma and LLC carcinoma cells. XPV was previously shown to be effective in inducing pronounced polyclonal immune responses specific to both melanoma- and carcinoma-associated antigens, and certain clinical efficacy was noted in some patients with stage IV melanoma and colorectal cancer [6, 7]. This report addresses the safety and immune efficiency of a lyophilized XPV regimen in stage III melanoma patients. In addition, we evaluated the overall five-year survival rate of XPV-treated patients.

Patients and methods

Patients

This study was performed in strict compliance with the protocol approved by the Scientific Council and Ethics Committee of the Institute of Clinical Immunology. Informed consent was obtained from every subject enrolled into this study. Eligibility criteria included: histologically proven measurable disease, no prior immunosuppressive therapy for a minimum of four weeks, good performance status (Karnofsky scale, 80% or more), adequate bone marrow, renal and hepatic functions, and no history of autoimmune disease.

Vaccine preparation

B16 melanoma and LLC carcinoma cells were grown in C57BL/6 (B6; H-2b) mice. When tumor nodules reached the desired size (diameter 1.6-2.0 mm), mice were euthanized and tumor tissues were harvested. Tumor cell suspensions were prepared by gentle pressing of tumor fragments in saline solution in a glass homogenizer at 4 °C. After washing, cells were stored at -20°C until lyophilization. Cell lysates were lyophilized and packaged in vials, such that each vial contained 50×10^6 B16 and 25×10^6 LLC lyophilized cells. Such doses were previously found to be immunogenic in cancer patients [6, 7]. After ionizing radiation sterilization, vaccine-containing vials were stored at-20 °C until use.

Vaccine administration

Immediately prior to administration, each vaccine dose was re-suspended in 2 mL saline solution. The induction course of vaccination consisted of 10 subcutaneous immunizations (five at weekly, and five at fortnight intervals) administered over a three-month period. Clinical application of the supporting vaccination schedule was dependent on the disease course and the patient's overall health status. The treatment was performed on an outpatient basis.

Toxicity assessment

Systemic toxicity was assessed according to the National Cancer Institute Common Toxicity Scale. Local vaccine toxicity was assessed according to the following scale: grade I, erythema and induration <20 mm in diameter; grade II, erythema and induration of 20 mm without ulceration; grade III, ulceration or painful adenopathy; and grade IV, permanent dysfunction related to local toxicity.

Blood cell analysis

Complete blood counts were performed using a Fully Automatic Cell Counter (Erma PCE-210, Erma Inc, Japan). Blood smears were stained with haematoxylin-eosin and analyzed according to standard protocols.

Biochemical analysis

Biochemical blood parameters were measured using an Automatic Biochemistry Analyzer (Furuno CA-180, Furuno Electric Company, Japan) and commercially available test-kits (Vector-Best; Kolzovo, Russia).

Flow cytometry

PBMCs were stained using commercially-available kits of Abs labeled with various fluorochromes (eBioscience, San Diego, CA). Relative frequencies of cells expressing CD3, CD4, CD8, CD16, CD20, CD45RO, and HLA-DR markers were analyzed on a flow cytometer (BD FACS Canto™ II, BD Biosciences, San Jose, CA) according to the manufacturer's instructions.

Proliferation assay

A human BRO melanoma is a tumor cell line capable of growing aggressively in nude mice [8]. BRO cells were grown as monolayers in RPMI 1640 medium supplemented with 10% FCS, 2 mM glutamine and antibiotics (all reagents from Sigma-Aldrich, St. Louis, MO). These cells were lysed by freeze-thawing [6, 7]. Peripheral blood mononuclear cells (PBMCs) at 2×10^5/well were cultured in the presence (test) or absence (control) of BRO cell lysates (5×10^4/well) in a 96-well round-bottomed plate (BDSL Laboratory Equipment, Ayrshire, UK) in triplicates for 5 days. Cell culture medium was RPMI 1640 supplemented with 5 mM HEPES, 2 mM L-glutamine, 5×10^{-5} M 2-mercaptoethanol, antibiotics and 10% autologous plasma. Cell proliferation was measured using a standard ^{3}H-thymidine incorporation assay and the results were expressed as mean counts per minute (cpm) of triplicate cultures. Stimulation index (SI) was calculated for each triplicate as follows:

$$SI = \left(\frac{\text{Mean}_{\text{CPM}} \text{ of lysate-stimulated wells}}{\text{Mean}_{\text{CPM}} \text{ of media-stimulated wells}} \right)$$

Serum assay

Immunoglobulins (Ig)A, IgM, IgG, antibodies (Abs) to dsDNA, rheumatoid factor, interferon-alpha (IFN-α), IFN-γ, tumor necrosis factor-α (TNF-α), interleukin-4 (IL-4), IL-6, IL-8, and IL-10 were measured in sera using an Automated EIA and Chemistry Analyzer (ChemWell® 2910, Awareness Technology, USA) and commercially available ELISA-kits, according to the manufacturer's instructions (Vector-Best; Kolzovo, Russia).

Statistics

Parametric Student's or non-parametric Mann-Whitney U test were used to compare immune-mediated effects. Kaplan-Meier survival analysis was employed to analyze overall survival curves and assess general mortality in the groups.

Results

Toxicity assessment

Locally advanced melanoma is known to have a limited toxic effect on the host. Therefore, this study of XPV-based treatment in melanoma patients provides objective and reliable information about XPV-related toxicity. In total, 41 stage III melanoma patients were subjected to XPV treatment at our Centre in 2008-2010. Twenty-seven patients (13 females and 14 males; median age 53) fulfilled the inclusion criteria (complete sets of toxicological, immunological and clinical follow-up data) and were included in the subsequent analysis, while 14 patients were excluded due to insufficient follow-up data. All patients had locally advanced cutaneous melanoma and underwent surgical excision of melanoma foci and metastases. No symptoms of grade III/IV systemic toxicity associated with the vaccine administration were documented during an induction course of XPV (10 vaccinations). Influenza-like syndrome (self-limiting sub-febrile temperature and musculoskeletal discomfort) was detected in 6/27 (22%) patients over 24 to 48 h post-vaccination. Local grade I/II toxicity symptoms (irritation at the injection site) were observed in 25/27 (92%) patients; such local manifestations were usually self-limiting and disappeared after 24 to 48 h. There were no cases of treatment-related hospitalizations or mortalities.

Hematopoiesis and immunopoiesis are known to rely on actively proliferating cells that are very sensitive to cytotoxic impacts. Therefore, potential XPV-mediated changes in blood cell frequencies can reliably characterize the toxicity of a novel XPV preparation at the cellular level. As shown in *table 1*, the XPV treatment did not significantly affect the number of red and white blood cells; similarly, relative percentages of CD3+, CD4+, CD8+, CD16+ and CD20+ lymphocytes were not affected in the blood samples of XPV-treated patients. Serum concentrations of Ig A, IgM and IgG were also not affected by XPV administration. No changes in erythrocyte sedimentation rate (ESR) nor in serum concentrations of rheumatoid factor and Ab specific to DNA were noted in XPV-treated patients (*table 1*). Similarly, we did not detect any significant XPV-mediated effects on serum concentrations of Abs to cardiolipin, thyroglobulin and microsomal fraction of thyrocytes (data not shown). Biochemical parameters of blood are known to characterize the functional activity of internal organs. As shown in *table 1*, no noticeable changes in these parameters were documented in patients after an induction course of vaccination, suggesting that XPV administration did not affect the functions of internal organs. Consistent with this data, XPV-treated patients did not exhibit any clinical evidence of the development of systemic autoimmunity.

Immune responses to XPV

The goal of cancer immunotherapy consists in the induction of memory T cells responsible for long-term anti-tumor protection. Following 10 XPV vaccinations, the patients exhibited relative increases in the number of memory CD4+CD45RO+ T cells, suggesting XPV-mediated induction of the sensitization phase of T-cell-mediated immunity to vaccine antigens (*figure 1*). However, it should be noted that no significant increases in CD8+CD45RO+ T cells were documented in those patients.

In order to characterize the influence of xenogeneic vaccination on T-cell responses to human melanoma-associated antigens in patients, we evaluated PBMC proliferative responses to BRO-associated antigens before and after the induction course of vaccination. We tested PBMC samples from 7 patients (3 females and 4 males; median age 52), which were available for this investigation and found that xenogeneic vaccination increased proliferative PBMC responses to BRO antigens. Indeed, SI before and after application of the induction vaccine course were 1.1 ± 0.4 and 2.9 ± 1.1, respectively ($p<0.05$), suggesting the development of considerable XPV-induced immune cross-reactivity to human TAAs in XPV-treated patients.

Immune cell-derived cytokines indicate the overall balance between pro-and anti-inflammatory cell-mediated responses occurring in the body. In this context, it is important that administration of the induction XPV course

Table 1. Blood parameters (M ± SEM) in melanoma patients (n = 27).

Parameter	Normal rate	Before XPV-based therapy	After an induction course of XPV-based therapy
Hemoglobin, g/L	120.0-160.0	131.0 ± 2.0	130.0 ± 2.0
Erythrocyte, × 10^{12}/L	4.5-5.5	4.2 ± .1	4.2 ± 0.1
Platelets, × 10^{9}/L	180.0-320.0	204.0 ± 19.0	225.0 ± 12.0
Leucocytes, × 10^{9}/L	4.0-8.0	5.6 ± 0.2	6.1 ± 0.3
Banded neutrophils, %	1.0-6.0	4.1 ± 0.5	3.2 ± 0,3
Segmented neutrophils, %	47.0-72.0	57.0 ± 2.0	56.0 ± 2.0
Basophils, %	0-1.0	0.1 ± 0.1	1.2 ± 0.2
Eosinophils, %	0-5.0	2.0 ± 0.3	3.0 ± 0.4
Monocytes, %	3.0-11.0	6.0 ± 1.0	6.0 ± 1.0
Lymphocytes, %	19.0-37.0	30.0 ± 2.0	32.0 ± 2.0
CD3 T lymphocytes, %	58.0-83.0	61.0 ± 2.0	60.0 ± 3.0
CD4 Tlymphocytes, %	29.0-59.0	35.0 ± 2,0	35.0 ± 2.0
CD8 T lymphocytes, %	17.0-40.0	24.0 ± 2.0	25.0 ± 2.0
CD4/CD8	0.9-2.8	1.5 ± 0.13	1.6 ± 0.17
CD20 B lymphocytes, %	8.0-17.0	10.0 ± 1.0	8.0 ± 1.0
CD16, %	6.0-24.0	20.0 ± 3.0	20.0 ± 3.0
Ig M, g/L	0.6-2.6	1.8 ± 0.31	2.0 ± 0.29
Ig A, g/L	0.7-3.8	2.1 ± 0.19	2.7 ± 0.42
Ig G, g/L	6.9-16.2	11 ± 0.6	14.3 ± 1.7
Erythrocyte sedimentation rate (ESR), mm/h	1.0-15.0	18.0 ± 3.0	16.0 ± 3.0
Anti-dsDNA Ab, U/mL	<40.0	25.0 ± 6.0	21.0 ± 5.0
Rheumatoid factor, U/mL	0-25.0	0.2 ± 0.2	0.3 ± 0.2
Protein, g/L	65.0-85.0	74.0 ± 1.0	76.0 ± 1.0
Bilirubin, μmol	3.5-20.5	12.0 ± 0.77	12.7 ± 1.17
Alanine aminotransferase (ALT), mckat/L	0-0.68	0.98 ± 0.28	0.37 ± 0.04
Aspartate aminotransferase (AST), mckat/L	0-0.68	0.53 ± 0.14	0.37 ± 0.04
Thymol test, U	0-4.0	3.9 ± 0.8	3.3 ± 0.6
Lactate dehydrogenase (LDH), U/L	225.0-450.0	364.0 ± 49.0	391.0 ± 54.0
Alkaline phosphatase, U/L	< 117.0	141.0 ± 37.0	138.0 ± 10.0
Urea, mmol	2.5-8.3	5.6 ± 0.3	5.8 ± 0.4
Creatinine, μmol	44.0-100.0	37.0 ± 10.0	49.0 ± 12.0
Fibrinogen, g/L	2.0-4.0	3.4 ± 0.20	3.8 ± 0.3

resulted in an increase in serum IFN-γ and IL-8 levels in 17 patients tested (nine females and eight males; age 42-68 years), suggesting up-regulation of pro-inflammatory cell activity in these patients. Interestingly, XCV-based treatment did not increase serum levels of IFN-α, TNF-α, IL-4, IL-6, and IL-10 (*table 2*).

Overall survival

Although the primary end points of this trial were toxicity and immune-mediated responses to XPV, we also evaluated the overall five-year survival in 27 XPV-treated patients with cutaneous melanoma. The control group was composed retrospectively of 27 patients who did not receive immunotherapy. To perform this case-control study, which matched clinical and prognostic parameters, each control patient was randomly selected to be clinically comparable to a particular XCV-treated patient (*table 3). Figure 2* shows that the overall five-year survival rate in the XPV-treated group was significantly higher compared to that in the control group (55 *vs*. 18%), suggesting XCV-mediated survival benefits in melanoma patients.

Discussion

An accumulating body of evidence suggests that xenogeneic cancer vaccines can be very effective in breaking the immune tolerance to low immunogenic human TAAs [4, 5]. It is also important that all humans possess natural (pre-existing) antibodies, which account for acute rejection of any non-primate cells. A significant proportion of these antibodies is represented by IgGs specific to an α-gal epitope abundantly expressed on glycoproteins and glycolipids in non-primate mammals [9]. By opsonizing

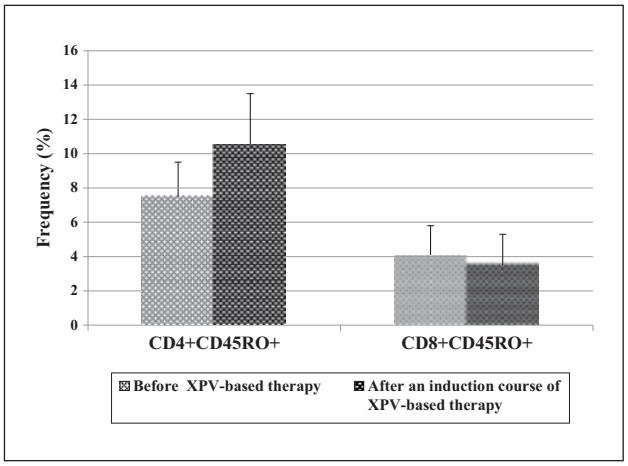

Figure 1. Frequency (M ± SEM) of memory CD4+CD45RO+ (left) and CD8+CD45RO+ (right) T cells in blood samples of patients before (light-grey columns) and after (dark-grey columns) an induction course of XPV-based immunotherapy.

Table 2. Concentrations (pg/mL, M ± SEM) of serum cytokines in melanoma patients (n = 17).

Cytokine	Before XPV-based therapy	After an induction course of XPV-based therapy
IFN-α	34.4 ± 35.6	43.0 ± 30.4
IFN-γ	33.2 ± 31.6	140.5 ± 133.3*
TNF-α	1.0 ± 0.14	4.3 ± 5.5
IL-4	9.4 ± 3.9	7.2 ± 4.5
IL-6	2.6 ± 1.37	9.0 ± 9.7
IL-8	17.5 ± 14.0	72.9 ± 77.6*
IL-10	3.5 ± 0.81	4.0 ± 1.3

** p<0.05, as compared to the appropriate levels before vaccinations*

Table 3. Characteristics of the patients assessable for survival.

Characteristic	Treatment group	Control group
Number of patients	27	27
Disease stage	III (T1-4NIM0)	III (1-4NIM0)
Males	14 (52%)	15 (55.6%)
Females	13 (48%)	12 (44.4%)
Age, years (median, range)	53.0 ± 1.8 (33-69)	53.1 ± 1.9 (35-72)

xenogeneic tumor cells, natural antibodies could promote internalization of antigenic material into antigen-presenting cells via an Fcγ-receptor-mediated mechanism, thus greatly enhancing the immunogenic cross-presentation of TAAs to tumor-specific T lymphocytes [4, 5].

A xenogeneic vaccine has been developed at our Centre that combines lysed murine B16 melanoma and LLC carcinoma cells. Significant increases in cell-mediated immune responses to both LLC and B16 antigens were documented in XPV-treated patients, as determined by delayed-type hypersensitivity skin reactions and blood lymphocyte proliferation responses. Importantly, XPV administration was

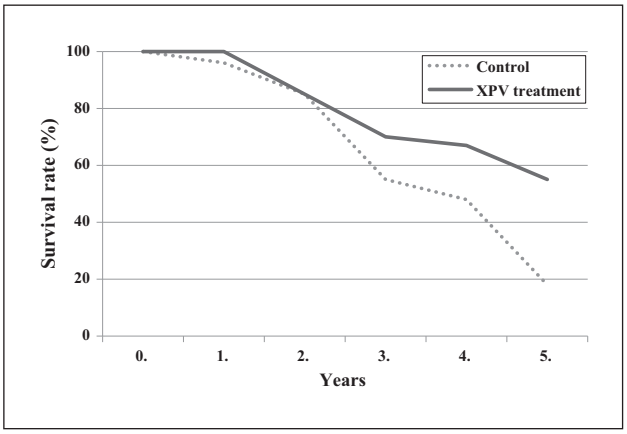

Figure 2. Survival rate of melanoma patients. The overall 5-year survival rate was assessed in 27 XPV-treated patients (solid line), as compared to 27 control patients who did not receive immunotherapy (dotted line). This case control study was performed using retrospectively selected control patients where each control patient was randomly selected to be clinically matched to a particular XCV-treated patient.

also capable of generating vaccine-specific IgG-mediated responses [6, 7]. The particular XPV variant used in the above-mentioned studies was not subjected to prior lyophilization. In the present study, we used a lyophilized XPV variant that was also characterized by an improved vaccine standardization protocol and a more convenient vaccine storage and handling protocol.

Data presented in this paper clearly indicates the safety of XPV administration. None of the XPV-treated patients experienced any clinically significant side effect. XPV-based treatment did not interfere with hematopoiesis, immunopoiesis and the functionality of internal organs. XPV-treated patients did not show laboratory and clinical evidence of XPV-mediated systemic autoimmunity, which could not be excluded, due to the broad range of xenogeneic antigens present in XPV. From the data obtained it appeared that XPV-based immunotherapy was not associated with the clinical complications frequently observed in other immunotherapeutic regimens, such as IFN-α-and IL-2-based approaches or Abs blocking immune checkpoint molecules [3]. Consequently, XPV-based therapy could be devoid of serious limitations and contraindications in its clinical application.

As demonstrated in the present study, XPV-based therapy led to a relative increase in the number of circulating memory CD4+CD45RO+ T cells. We propose that a significant proportion of XPV-induced memory T cells could be cross-reactive with human differentiation antigens present in the tumor. Consistent with this hypothesis, PBMCs isolated from XPV-treated patients exhibited noticeably increased proliferative responses to human BRO melanoma-associated antigens. Compared to naïve T cells, memory T cells are far less sensitive to tumor-mediated immunosuppression and thus could remain functionally active even in patients with advanced cancer [10, 11]. Of great interest is whether or not the XPV is able to induce immune responses directed against the patient's own tumor. With vaccinations, an increase in T-cell mediated responses to self melanoma-associated antigens was

observed by us in 3 out of 6 assessable patients with disseminated (stage IV) melanoma [6 and our own unpublished data]. Unfortunately, we were unable to evaluate immune responses to self-melanoma-associated antigens in other patients because autological tumor material was not available.

XPV administration augmented the serum levels of IFN-γ and IL-8 in melanoma patients without significantly affecting levels of TNF-α, IL-4, IL-6 and IL-10. This data suggests an increased functional activity of T helper-1(Th1) cells, which are believed to be pivotal in immune-mediated antitumor responses, as compared to Th2 cells [12, 13]. Our previous data showed up-regulation of both Th1-and Th2-type cytokines (IFN-γ and IL-4) in XPV-treated patients [6, 7], which is different from the strictly Th1-type cytokine bias detected in the present study. We speculate that this discrepancy could result from a higher pro-inflammatory activity of the freeze-dried XPV vaccine preparation in comparison with its non-lyophilized analogue used in earlier studies.

Although the primary endpoints of this study were toxicity and immune-mediated responses to XPV, we evaluated the overall 5-year survival rate for XPV-treated melanoma patients. The results indicated significant survival benefits of XPV-based immunotherapy for patients with locally advanced melanoma. As with other immunotherapies, the XPV-based therapy could be maximally effective when applied as early as possible following surgical excision of the primary tumor and local metastases. It is of great importance that patients in receipt of XPV can lead an active life and can work at full capacity.

In conclusion, this study showed the safety, immune efficiency and clinical feasibility of XPV-based immunotherapy application following surgical excision of locally advanced melanoma. In principle, we believe that, when administered at early disease stages, xenogeneic vaccine therapy could become an optimal regimen for the prevention of melanoma recurrences, as well as for other cancers. From the technological standpoint, it is important that a wide variety of animal tumor cell lines are available, which could be exploited to design other XPV vaccines that would ensure maximal antigenic overlap with the target tumors in humans. Such vaccines could potentially elicit strong polyclonal tumor-specific immune responses with minimal immune-based side effects. ■

Disclosure. *Financial support: his study was supported by the Neurotechnology company (Vilnius, Lithuania). Conflict of interest: none.*

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