Minimally invasive diagnostics and immunotherapy of lung cancer

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Chapter 8

Phase I Study: safety and immunogenicity of vaccination with XAGE-1b long peptides combined with poly-ICLC in patients with stage IIIb/IV pulmonary adenocarcinoma

Adapted study protocol with preliminary results
(Protocol ID: NL44189.000.13)

INTRODUCTION

Lung cancer

Lung cancer is the most common cause of cancer mortality in men in the developed world and one of the leading causes in women[1]. The two major forms of lung cancer are non-small cell lung cancer (NSCLC) and small cell lung cancer (SCLC). NSCLC comprises about 80 % of all lung cancers[2]. NSCLC patients are staged according to the TNM classification system[3-5]. Patients with stage I and stage II non-small cell lung cancer are, when operable, treated with complete surgical resection[6]. Patients with stage III disease are treated with combined chemo-radiotherapy[7]. In the LUMC, stage III adenocarcinoma patients are generally treated for 25 working days by a combination of daily shots of cisplatin followed by radiotherapy. Patients with stage IV disease are treated with palliative chemotherapy[8]. In the LUMC, stage IV NSCLC patients are generally treated with three schemes of chemotherapy (one scheme lasting 21 days) depending on the histology of the primary tumor; squamous cell carcinoma is treated by a combination of carboplatin and gemcitabine, whereas adenocarcinoma is treated by a combination of carboplatin and pemetrexed.

The five-year survival for lung cancer patients is poor. This can in part be explained by the fact that the majority of lung cancer patients present with advanced disease (stage III/IV). For NSCLC patients, the 5-year survival is 14%[2,9]. The medical need for new treatment strategies for lung cancer is therefore high. New anti-cancer therapies have been advocated in recent years, especially for stage IV lung cancer patients. For example, tyrosine kinase inhibitors (e.g. Iressa, Tarceva) have been shown to have the potential of long term suppression of tumor activity in patients with lung adenocarcinoma who have a somatic mutation in the Epidermal Growth Factor Receptor (EGFR) gene[10]. Immunotherapy with therapeutic vaccines targeting specific tumor antigens is another promising treatment strategy for lung malignancies.

Immunotherapy using synthetic long peptides

The immune system plays a major role in cancer. Professional antigen presenting cells (APC), mostly dendritic cells (DC), sample antigen that is secreted or shed by tumor cells. The tumor antigens are processed and presented as peptides by major histocompatibility complex (MHC) class I and II molecules on the DC surface, and recognized by the T-cell receptor (TCR) of T-cells. Major histocompatibility complex (MHC) class I – peptide complexes are recognized by CD8+ T-cells that become activated and differentiate into cytotoxic T-lymphocytes (CTL). MHC class II – peptide complexes are recognized by the TCR of the CD4+ T-helper (Th) cells. Upon stimulation, tumor-specific CD4+ T-cells deliver essential help for tumor-specific CD8+ T-cells by fully activating DC through the CD40-CD40L signaling pathway as well as by secretion of IL-2[11]. Moreover, the tumor-specific

CD4+ T-cells provide help by guiding the homing of CD8+ T-cells and by polarizing the local tumor environment to allow tumor-specific CD8+ T cells to exert their tumoricidal function[12]. In the ideal situation, the close interaction between DC, CTL and Th cells will lead to control of tumor growth and ultimately tumor regression.

At the LUMC, we are currently developing a therapeutic vaccination strategy against HPV16 and p53 expressing malignancies making use of synthetic long peptides (SLP) based on the associated tumor antigens. In experimental models, SLP-based vaccines have shown to be superior to protein vaccines as they induce stronger CTL responses in a direct comparison[13] as well as over viral vector vaccines[14]. The SLP-based vaccines used so far covered non-viral (p53) as well as virus-encoded tumor antigens (HPV-16 E6/E7) and these have turned out to be safe in patients and induced robust T-cell responses in several early phase clinical trials in patients with malignant disease[15-17]. Moreover, vaccination of patients with HPV-16 induced high-grade (premalignant) vulvar intraepithelial neoplasia (VIN 3) with an HPV16-based SLP resulted in complete and durable regressions of VIN 3 lesions[18]. Importantly, a clear link was established between the kinetics and phenotype of the immune response (strong and broad vaccine-prompted HPV16-specific proliferative response with higher IFNγ and IL-5 levels) and complete regression of the lesion[19]. These results showed for the first time that immunotherapy against existing neoplastic lesions by therapeutic vaccination can be truly effective in human beings.

Rationale of XAGE-1b as target for long peptide vaccination in lung cancer

A prerequisite for immunotherapy of lung cancer with SLP-based vaccination is the identification of suitable tumor antigens that provide an immunological window for immunotherapy. Tumors express antigens that – when presented by DCs – can elicit adaptive immune response aimed at eradicating the primary tumor. These so-called tumor antigens can be classified in three categories: 1) antigens that are overexpressed in tumors; 2) antigens that are of viral origin; and 3) antigens that are specifically expressed in several different tumors[11,14].

An interesting group of tumor antigens, frequently recognized by the immune system of cancer patients due to their restricted expression pattern, is encoded by 'cancer-testis'(CT) genes. These CT antigens are present in a significant subset of tumors, including NSCLC. XAGE-1 is a member of the family of CT antigens. The XAGE-1 protein has 4 transcripts (a, b, c and d), of which XAGE-1b (81 amino-acids) is the isoform that is mainly expressed [20-22]. Using a monoclonal antibody against XAGE-1b (USO9-13), immunohistochemical nuclear staining has been observed in 25/47 lung adenocarcinomas, 1/12 hepatocellular carcinomas and 1/11 gastric cancers, but not in adjacent normal tissues[22], indicating highly preferential expression of XAGE-1b in lung adenocarcinoma, a subtype that accounts for 40% of NSCLC. Our preliminary data, using the same monoclonal

antibody (USO9-13), confirm the percentage of XAGE-1b expression in lung adenocarcinoma. Staining of paraffin-embedded resected lung adenocarcinoma tissue revealed positive staining in 4 out of 8 patients (50%). An association of XAGE-1b protein expression in lung adenocarcinomas with clinical prognosis has been suggested[20]. Circulating antibodies generated against XAGE-1b have been observed in 10% of patients with lung adenocarcinoma[21]. The presence of XAGE-1b-specific antibodies is indicative for an underlying XAGE-1b-specific CD4+ T-cell response. In addition, it has been shown that full-length XAGE-1b protein-pulsed human DCs are able to induce specific CTL responses in vitro[23]. These data suggest that (1) there is no immune tolerance against XAGE-1b at the CD4+ and CD8+ T-cell level; (2) the XAGE-1b amino acid sequence comprises CD4+ and CD8+ T-cell epitopes; and (3) this protein is sufficiently immunogenic to spontaneously trigger an immune response in patients.

To confirm these results in a Dutch population, a preclinical study was conducted to detect immune responses against XAGE-1b. Overexpression of XAGE-1b was found in 17 of 39 (43.6%) pulmonary adenocarcinomas by immunohistochemistry. In 20 patients, analysis of T-cells isolated and expanded from the primary tumor and its draining lymph node demonstrated XAGE-1b specific responses in 2 patients. XAGE-1b-specific IgG antibodies (detected by ELISA) were found in serum from 3 out of 40 patients (7.5%). All three antibody positive patients also displayed a systemic T-cell response to XAGE-1b, measured by proliferation, cytokine production and upregulation of T-cell activation markers CD137 and CD154 and intracellular expression of IL-2 and IFN-γ in peripheral blood mononuclear cells (PBMCs). Subsequently established T-cell clones from these bulk cultures showed strongest proliferation against XAGE-1b peptide 1 (amino acid 1-32) and peptide 2 (amino acid 18-42), the N-terminal part of the XAGE-1b protein[24].

These findings are in line with a recent study on the spontaneous immune response against XAGE-1b in non-small cell lung cancer (NSCLC) patients. An antibody response against XAGE-1b was observed in 10% (20/200) of NSCLC patients and in 19% (13/69) of stage IIIB/IV lung adenocarcinoma patients. A CD4+ T-cell response was detected in 88% (14/16) and a CD8+ T-cell response in 67% (6/9) in the XAGE-1b antibody-positive patients examined[23,25].

Rationale of Montanide ISA 51 VG and Hiltonol® as adjuvants to a XAGE-1b SLP vaccine

Therapeutic long peptide vaccines can be improved by adding adjuvants. These are an important component of vaccine formulations since they can enhance immune responses[26]. Montanide ISA 51 VG is defined as a mixture of a highly purified mineral oil (Drakeol 6VR) and a surfactant (Mannide monooleate). When mixed with an aqueous phase in a 50/50 ratio, it renders a water in oil emulsion. This water-in-oil emulsion is frequently used as an adjuvant in numerous clinical trials[27-30]. Most common local reactions

are local pain, tenderness, erythema and granuloma at the injection site. In a less extent, indurations and swelling are described[26]. Trials performed by our group combining a SLP vaccine with Montanide ISA 51 VG induced vaccine peptide specific T-cell responses[15,17]. However, results from the first two trials with p53-SLP showed that vaccine-induced Type 1 T-helper cells produced only low amounts of the key cytokines such as IFN-γ and IL-2, indicating that these responses, although present, were not properly polarized by Montanide ISA 51 VG alone[16,31].

Therefore, to benefit from the tumor-specific T-cells at the tumor site, peptide vaccines should be combined with immune modulating adjuvants that specifically induce polarization Th1/CTL response. This can be achieved by using Toll-like receptors (TLR) agonistic compounds[14,26]. TLR are a class of proteins that play a key role in the innate immune system. These receptors recognize conserved molecules derived from microorganisms and can subsequently induce immune responses[32]. TLR3 is the specific intracellular recognition system that responds to the intracellular presence of RNA virus infection. It recognizes and signals in response to the intracellular presence of dsRNA intermediates, resulting in a local cytokine burst[33]. Polyribosinic:polyribocytidic acid (Poly-IC) is a TLR3 agonist which has been to shown to induce DC maturation and to mediate the release of IFNγ, IL-4, IL-6 and IL-12[34,35]. In a PSA-transgenic mouse model, it could augment both cellular and humoral immune responses to PSA and PSA-anti-PSA immune complex[34]. Importantly, Hiltonol® (Oncovir Inc., Washington DC), a modified poly-IC stabilized with polylysine (poly-ICLC), has been tested and shown to be safe in several recent immunotherapy clinical trials, including studies in which it was combined with antigenic or peptide-based vaccination[36,37].

Montanide ISA 51 VG and Hiltonol® have also been combined. Recently, results were reported of a phase I trial in which ovarian cancer patients were treated with another CT antigen based SLP vaccine (NY-ESO-1 overlapping peptides (OLP)) using Montanide ISA 51 VG and Hiltonol® as adjuvants. The vaccine was generally well tolerated and was not associated with a high rate of severe adverse events (SAE), laboratory abnormalities, abnormalities in vital signs and physical examinations, or decrease in Karnofsky performance status. Most notably, the combination of vaccine, Montanide ISA 51 VG and Hiltonol® displayed the best capacity to induce NY-ESO-1 specific immune responses in patients as opposed to OLP vaccine only or OLP vaccine with Montanide ISA 51 VG[36].

In conclusion, XAGE-1b is an attractive target for immunotherapy due to its preferential expression in lung adenocarcinoma and due to its potential to elicit spontaneous humoral and cellular immune responses in patients with adenocarcinoma. Furthermore, adding Montanide ISA 51 VG and Hiltonol® as adjuvants to a peptide vaccine can help boost antigen specific immune responses. Here, we describe a phase I clinical trial designed to evaluate the safety and immunogenicity of XAGE-1b SLP vaccine combined with Montanide ISA 51 VG and Hiltonol® in patients with lung adenocarcinoma.

OBJECTIVES

Primary objective:

– To evaluate the safety of vaccination with XAGE-1b peptides emulsified in Montanide ISA 51 co-mixed with the adjuvant Hiltonol® (Poly-ICLC) in patients with stage IIIb/IV pulmonary adenocarcinoma.

Secondary objective:

– To evaluate the capacity of the vaccination strategy to induce XAGE-1b-specific humoral and cellular immune responses in lung adenocarcinoma patients, including the migratory capacity of XAGE-1b vaccine-induced T-cells into the vaccine injection site.

STUDY DESIGN

This uncontrolled exploratory phase I study will be performed in 30 patients with stage IIIb/IV primary pulmonary adenocarcinoma patients, who have been previously treated at the department of Pulmonology with combined chemo-radiotherapy (stage III) or with chemotherapy alone (stage IV). An overview of this phase I trial is presented in **Figure 1**.

After obtaining informed consent, patients will be screened for eligibility in the study. The patients will receive 4 vaccinations consisting of 5 synthetic overlapping peptides, covering the entire XAGE-1b protein, emulsified in Montanide ISA 51 and co-mixed with the adjuvant Hiltonol®, by subcutaneous injection in one of the arms. Vaccinations will be given at three week intervals, each at another limb. The first 2 groups of 5 patients (stage IV and stage IIIb adenocarcinoma) will be vaccinated with a peptide dose of 50 mg together with an adjuvant dose of 1 mg. The groups will start simultaneously. In the next 2 groups of 5 patients (stage IV and stage IIIb adenocarcinoma), the dose of peptides will be simultaneously increased to 150 mg together with an adjuvant dose of 1 mg. The last 2 groups of 5 patients (stage IV and stage IIIb adenocarcinoma) will be simultaneously vaccinated with a peptide dose of 300 mg together with an adjuvant dose of 1 mg. A schedule of events per study patient is displayed in **Figure 2**.

Inclusion criteria

For stage IV pulmonary adenocarcinoma patients:

– Histologically proven pulmonary adenocarcinoma stage IV or stage IIIb according to recent guidelines on TNM classification of NSCLC[4]
– Age ≥ 18 years

- Completion of standard (platinum-based) chemotherapy schedules or combined chemo-radiotherapy schedule (concurrent or sequential)
- No intention for further chemotherapy treatment
- Good WHO performance status (0-2)
- Adequate bone marrow function: WBC ≥ 2.0 x 10^9/l, platelets > 100 x 10^9/l, hemoglobin > 5.0 mmol/L
- Patients of child-bearing potential should test negative using a serum pregnancy test
- Survival expectation > 3 months

Exclusion criteria

- Candidates eligible for restaging and curative surgical resection of the primary tumor after good response to treatment with chemo-radiotherapy
- Progressive disease after finishing standard chemotherapy or chemo-radiotherapy schedule
- Inadequate bone marrow function more than 3 weeks after last chemotherapy treatment.
- Poor WHO performance status (3-5)
- Eligibility for treatment with Tyrosine Kinase Inhibitors (e.g. erlotinib)
- History of an autoimmune disease or other systemic intercurrent disease that might affect the immunocompetence of the patient, or patients receiving immunosuppressive therapy including transplant recipients
- Second primary tumor of non-pulmonary origin
- CD4 cell count < 200/m3 at baseline
- Known seropositivity for Hepatitis B Virus and/or HIV
- History of serious liver or kidney dysfunction, heart condition or thyroid disorder
- Receipt of another investigational product within the previous 4 weeks or at any time during the study period.
- Medical or psychological condition which in the opinion of the treating chest physician and investigator would not permit the patient to participate in or to complete the study

METHODS

Safety will be assessed during the whole study by collecting all adverse events according to the Common Terminology Criteria (CTC) version 3 and by monitoring vital signs, blood chemistry and hematological parameters[38]. A total of 250 ml of fresh blood will be collected from all patients at baseline (100ml), after 6 weeks (50 ml) and after 12 weeks (100ml). Immunological responses will be monitored using PBMCs that are tested

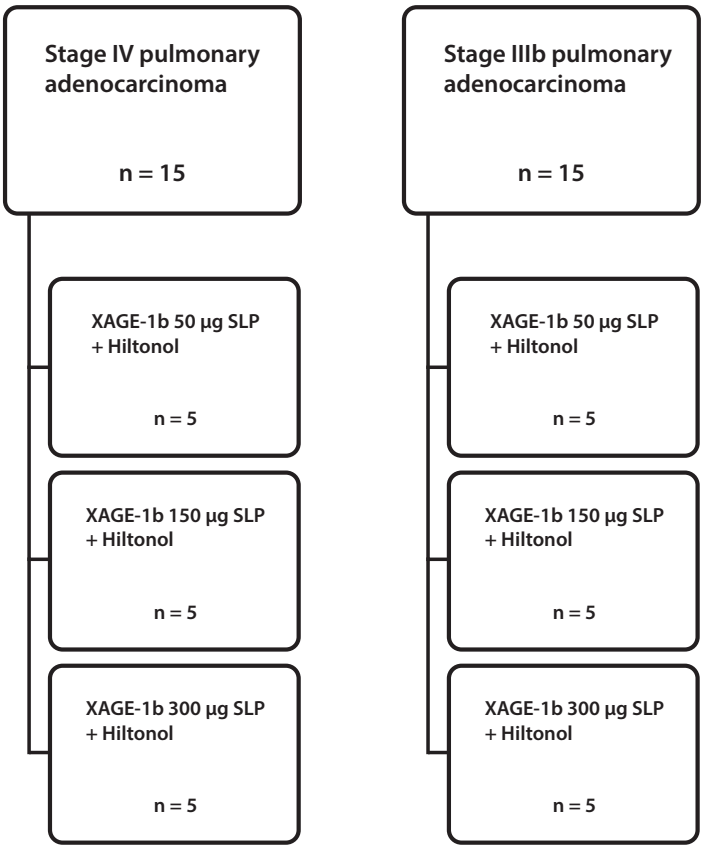

Figure 1. Overview of phase I study. In a dose-escalating fashion (50-150-300 µg), thirty pulmonary adenocarcinoma patients (15 stage IIIB and 15 stage IV) will be given a subcutaneous injection of a vaccine consisting of 5 overlapping peptides covering the entire XAGE-1b protein emulsified in Montanide ISA 51 and co-mixed with Hiltonol® (Poly-ICLC). Safety and immunogenicity of the vaccine will be assessed. Abbreviation: SLP synthetic long peptides

by IFNγ-ELISPOT and intracellular IFNγ/IL-2 staining for directly ex-vivo detection and enumeration of antigen-specific CD4+ and/or CD8+ T-cells, as well as following one round of in vitro stimulation. In addition, proliferation (lymphocyte stimulation test: LST) and associated cytokine production (IFNγ, TNFa, IL-4, IL-5, IL-10, and IL-2) will be assessed. Furthermore, a skin biopsy of the last vaccination site will be used to assess the migratory capacity of vaccine-induced T-cells. Finally, we will collect blocks of paraffin embedded tumor tissue of all patients in order to study the expression of XAGE-1b and other associated tumor antigens.

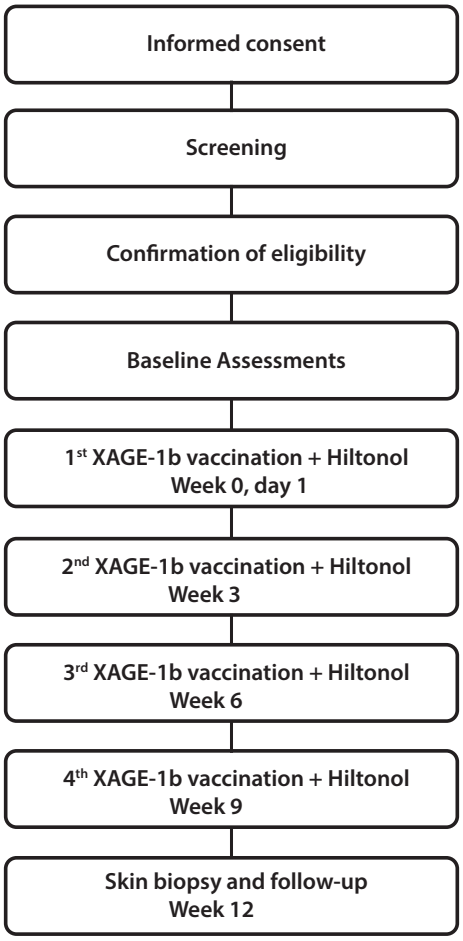

Figure 2. Schedule of events. Adverse events will be monitored throughout the study. For immunomonitoring, blood samples are collected at day 1, 22 and 43, and a skin biopsy of the last vaccination site at week 12.

Immunomonitoring of local changes

A biopsy sample of the last vaccination site obtained 3 weeks after vaccination will be used to monitor the migration of the vaccine-induced T-cells towards antigen-expressing lesions. T-cells will be cultured out of the biopsy and expanded by exposing them to a cytokine cocktail (TCGF, IL-7 and IL-15) as previously described[39] and tested using the same assays as described below for the monitoring of blood samples.

Immunomonitoring of systemic changes

In order to assess the induction of systemic T-cell immunity against XAGE-1b by the vaccinations, the PBMCs in the collected blood samples will be examined for proliferation (LST), cytokine production (IFNγ, TNFα, IL-4, IL-5, IL-10, and IL-2) as well as by

ELISPOT (IFNγ); a set of complementary and qualified assays for ex-vivo detection of antigen-specific responses. Multiparameter flow cytometry will be used to determine the phenotype (CD4+ and/or CD8+) and the percentage of activated XAGE-1b-specific T-cells in combination with IFNγ and IL-2 production, both directly ex-vivo and after one round of in vitro stimulation.

(1) XAGE-1b peptide-specific proliferation assay and cytokine bead array

XAGE-1b-specific proliferation will be assessed using a short-term (6-day) assay in combination with cytokine production by cytokine bead array (IFNγ, TNFα, IL-4, IL-5, IL-10, and IL-2) on supernatants taken at day 6. This combined assay will be performed according to described SOPs and has been published[16,17,19,40]. The average and standard deviation of the 8 medium only control wells are calculated and the cut-off was defined as this average plus 3xSD. The stimulation index (SI) is calculated as the average of 8 test wells divided by the average of the 8 medium control wells. A positive proliferative response is defined as a stimulation index of at least 3 and the counts of at least 6 out of the 8-wells above the cut-off value[40]. A vaccine-induced response is defined as a SI which is at least 3-fold higher than the pre-existing response[17,19]. Positive antigen-specific cytokine production is defined as a cytokine concentration above the detection limit (i.e. 20 pg/ml) and at least 2x the concentration of the medium control. A vaccine-induced response was defined as at least a 3-fold increase in the antigen-specific cytokine production before vaccination.

(2) XAGE-1b peptide-specific IFNγ-ELISPOT for CD4+ T-cells

IFNγ 4-day ELISPOT is our standard assay for measuring (tumor) antigen-specific T-cell responses and will be performed according to described SOPs and has been published[16,17,19]. Spots are counted with a fully automated computer-assisted-video-imaging analysis system (BioSys 5000). Specific spots are calculated by subtracting the mean number of spots in quadruplicate wells + 2xSD of the medium only control from the mean number of spots in experimental wells. Antigen-specific T-cell frequencies are considered to be increased compared to non-responders when specific T-cell frequencies are ≥ 1/10,000. T-cell frequencies are considered to be boosted by the vaccine when they were at least 3-fold higher than those prior to vaccination.

(3) Multiparameter analysis of XAGE-1b-specific T-cells by flow cytometry

This assay measures the percentage and polarization of XAGE-1b-specific T-cells. Peripheral blood lymphocytes, which respond to peptide stimulation in vitro, will be simultaneous stained for the T-cell markers (CD3, CD4 and CD8), activation markers (CD137 and CD154) and cytokines (IFNγ, IL-2) followed by acquisition and analysis by a flow cytometer according to described SOPs as has been published before[16,41]. The presence of XAGE-1b-specific T-cells is considered to be positive when the percentage

of XAGE-1b-peptide stimulated CD4+CD154+CD137+ (double activation markers) or CD8+CD137+ (single activation marker) T-cells is at least twice the percentage detected in the medium only control, and the responding cells should be visible as a clearly distinguishable population in the analysis dotplot. The percentage of IFNγ and/or IL-2 producing XAGE-1b-specific T-cells is determined by gating on the activated T-cell population. A vaccine-induced reaction is defined as at least a 3-fold increase in the percentage of antigen-specific T-cells compared to prior to vaccination. Both directly ex-vivo detection and enumeration of antigen-specific CD4+ and/or CD8+ T-cells, as well as following one round of in vitro stimulation, will be assessed.

(4) ELISA to detect vaccine induced XAGE-1b specific humoral responses

Serum samples (obtained at different time-points before, during and after vaccination) are subjected to a XAGE-1b peptide ELISA for detection of XAGE-1b peptide-specific immunoglobulin G (IgG). A 96-well plate (Costar 3590) is coated overnight at 4°C with the individual XAGE-1b peptides (50 µl of 5 µg/ml diluted in 0.1 M carbonate/bicarbonate coating buffer; Merck, Darmstadt, Germany). Then, the plate is washed six times with phosphate buffered saline (PBS; Fresenius Kabi Bad Homburg, Germany) and blocked for 1 h at room temperature (RT) in 100 µl/well PBS+5% FCS (fetal calf serum), which is assigned as blocking buffer. After six washings with PBS, the serum samples diluted in blocking buffer (1:100) are added to triplicate wells (50 µl/well) and incubated at RT for 2 h. As a positive control, a previously obtained serum sample with high XAGE-1b IgG titer (spontaneously induced) will be used. Subsequently, the plate is washed again and 50 µl/well of goat anti-human IgG-Horseradish peroxidase (HRP; Southern Biotechnology, Birmingham, AL) (diluted 1:3,000 in blocking buffer) is added and incubated for 1h at RT. Finally, after the six washings tetramethyl-benzidine liquid substrate (50 µl/well TMB, Sigma Aldrich) is added for the colorimetric enzymatic reaction. This reaction is stopped by adding 50 µl/well of 2 M H_2SO_4 (Merck) and the plate will be read in an ELISA reader at 450 nm.

The average OD-value of the triplicate uncoated wells (background value) is calculated and values are discarded if these are more than 2SD above this average. A positive XAGE-1b peptide specific IgG response is defined as at least a 2-fold increase compared to the background value. A 2-fold increase of the post-vaccination serum sample over that of the pre-vaccination serum sample is considered a *vaccine-induced* positive XAGE-1b peptide specific IgG response.

Statistical Analysis

To assess the secondary endpoint we will use the Fisher's exact test to analyze the response rate of the patients reacting to XAGE-1b pre- and post-vaccination among all patients (n=30) as well as among stage IIIb (n=15) or stage IV patients (n=15), irrespective of the dose level. The Chi-square test will be used to analyze potential differences in

the response rate of the patients (stage IIIb and IV combined) at each dose level (n=10 per dose level). The response rate will be analyzed for all 4 indicated immune tests separately. To assess differences in the strength of the response, which is defined as the breadth and the magnitude of the measured immune response within one test group, we will use the median specific spot count (ELISPOT), median stimulation index (LST), or median amount of cytokine production (CBA) obtained for all 5 different peptides per patient, of all patients in one dose group, using the nonparametric Mann – Whitney test with use of GraphPad InStat software, version 6.00 or higher. All reported P values will be two-sided and will not be adjusted for multiple comparisons. $P < 0.05$ is considered to indicate statistical significance.

TRIAL STATUS AND PRELIMINARY RESULTS

Patients are being recruited for this phase I study and currently one patient has been included and has finished his vaccination schedule. The results of LST assay of this patient are shown in **Figure 3**. A vaccine-induced proliferative response (SI index >3, indicated by asterisks) to XAGE-1b peptides X2, X3 and X4 was observed after the first vaccination, but not after the second vaccination. This patient demonstrated a strong response throughout vaccination to influenza matrix 1 protein-derived (Flu) peptides and memory response mix (MRM), which are common viral and bacterial recall antigens respectively. These strong recall antigen T-cell responses reflect an adequate general immune status of this patient.

Furthermore, another 2 patients have been included who have not yet finished their vaccination scheme.

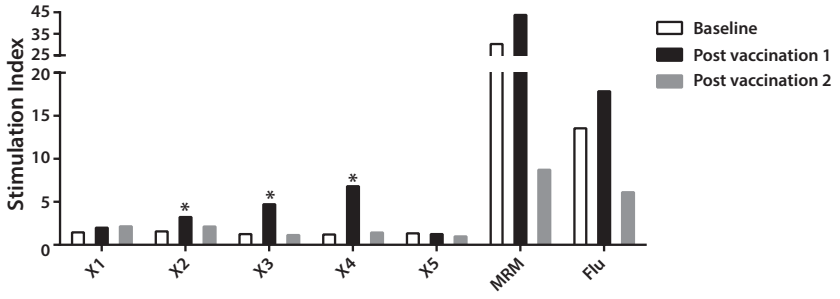

Figure 3. Results from the lymphocyte stimulation test (LST) from the first vaccinated patient (X-VAC-001). A XAGE-1b specific vaccine-induced proliferative response (SI index >3, indicated by asterisks) was observed after the first vaccination, but not after the second vaccination. Flu (influenza matrix 1 protein-derived) peptides and MRM (memory response mix) are common viral and bacterial recall antigens respectively, both of which serve as positive control and as parameters of general adaptive immune status of the patients.

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