Long-lasting Disease Stabilization in the Absence of Toxicity in Metastatic Lung Cancer Patients Vaccinated with an Epitope Derived from Indoleamine 2,3 Dioxygenase

Trine Zeeberg Iversen, Lotte Engell-Noerregaard, Eva Ellebaek, Rikke Andersen, Stine Kiaer Larsen, Jon Bjørn, Claus Zeyher, Cécile Gouttefangeas, Birthe Moerk Thomsen, Bente Holm, Per thor Straten, Anders Mellemgaard, Mads Hald Andersen, and Inge Marie Svane

Abstract

**Purpose:** To investigate targeting of indoleamine 2,3 dioxygenase (IDO) enzyme using a synthetic peptide vaccine administered to patients with metastatic non–small cell lung cancer (NSCLC).

**Experimental Design:** In a clinical phase I study, we treated 15 HLA-A2–positive patients with stage III–IV NSCLC in disease stabilization after standard chemotherapy. Patients were treated with imiquimod ointment and subcutaneous vaccinations (100 µg IDO5 peptide, sequence ALLEIASCL, formulated in 900 µL Montanide). Primary endpoint was toxicity. Clinical benefit and immunity were assessed as secondary endpoints.

**Results:** No severe toxicity was observed. One patient developed a partial response (PR) after one year of vaccine treatment, whereas long-lasting stable disease (SD) ≥ 8.5 months was demonstrated in another six patients. The median overall survival (OS) was 25.9 months. Patients demonstrated significant improved OS (P = 0.03) when compared with the group of patients excluded because of HLA-A2 negativity. IDO-specific CD8 T-cell immunity was demonstrated by IFN-γ Elispot and Tetramer staining. Fluorescence-activated cell sorting analyses demonstrated a significant reduction of the Treg population (P = 0.03) after the sixth vaccine (2.5 months) compared with pretreatment levels. Furthermore, expression of IDO was detected in nine of ten tumor biopsies by immunohistochemistry. High-performance liquid chromatography analyses of kynurenine/tryptophan (Kyn/Trp) ratio in sera were performed. In long-term analyses of two clinical responding patients, the ratio of Kyn/Trp remained stable.

**Conclusions:** The vaccine was well tolerated with no severe toxicity occurring. A median OS of 25.9 months was demonstrated and long-lasting PR + SD was seen in 47% of the patients. Clin Cancer Res; 20(1); 221–32. © 2013 AACR.

Introduction

Lung cancer is one of the leading causes of cancer deaths in both men and women worldwide, with non–small cell lung cancer (NSCLC) accounting up to 85% of the cases (1). At the time of diagnosis, most patients present with inoperable, advanced stage III-IV disease, with poor prognosis and a 5-year survival rate of less than 5%. Immunotherapy has the ability to activate the host’s cytotoxic CD8 T cells, and these immune cells might infiltrate the tumor and mediate elimination of cancer cells. Thus, therapeutic cancer vaccines have the potential to induce long-lasting, tumor-specific immune memory although in terms of treating metastatic cancer results have been somewhat disappointing. Nonetheless, therapeutic vaccines such as tumor cell vaccine and antigen-specific vaccines have shown clinical efficacy in early trials and are currently under clinical investigation in large, phase III randomized trials for adjuvant and metastatic settings in patients with NSCLC (2). Immune checkpoint blockade such as anti-CTLA-4 antibody is an anticancer therapy of current interest in NSCLC due to the shown inductions of T-cell activation and clinical activity (3). Other checkpoint blockade treatments like the antibody against programmed death 1 (PD-1) and its ligand (PD-L1) have shown clinical responses in patients with advanced NSCLC in early-phase I clinical trials (4, 5). These data support the idea that checkpoint blockade can enhance polyfunctional spontaneous preexisting T-cell responses.

**Authors’ Affiliations:** 1Center for Cancer Immune Therapy, Department of Haematology & Department of Oncology, Copenhagen University Hospital at Herlev, Herlev, Denmark; 2Department of Pathology, Bispebjerg Hospital, Team Bispebjerg (RH), Bispebjerg Bakke, Copenhagen NV, Denmark; 3Interfaculty Institute of Biochemistry; and 4Department of Immunology, Interfaculty Institute of Cell Biology, University of Tübingen, Tübingen, Germany.

**Note:** Supplementary data for this article are available at Clinical Cancer Research Online (http://clincancerres.aacrjournals.org/).

**Corresponding Author:** Inge Marie Svane, Center for Cancer Immune Therapy (CCIT), Copenhagen University Hospital at Herlev, Herlev Ringvej 75, 2730 Herlev, Denmark. Phone: 45-3868-3868; Fax: 45-4488-4153; E-mail: Inge.Marie.Svane@regionh.dk

doi: 10.1158/1078-0432.CCR-13-1560

©2013 American Association for Cancer Research.
Translational Relevance

Indoleamine 2,3-dioxygenase (IDO) potently inhibits T-cell immunity in patients with cancer. Blocking of this negative regulation pathway might promote immune-mediated tumor regression. In this first in-man clinical study (NCT01219348), we demonstrate clinical relevance of targeting IDO by a peptide vaccine strategy in 15 metastatic, stage III/IV non–small cell lung cancer (NSCLC) patients. We show that IDO is frequently expressed in lung adenocarcinomas hence representing an attractive target for cancer immunotherapy. Clinical evaluation demonstrated one patient with a partial response induced and another six patients eliciting long-lasting disease stabilization. The median OS was 25.9 months with no severe toxicities observed. Treg level was reduced during treatment and IDO-specific CD8+ T cells from treated patients were able to kill IDO-expressing cancer cells. Lysis of cancer cells while suppressing regulatory T-cell function might be important for clinical benefit and therapeutic targeting of IDO may be an important vaccine strategy in metastatic NSCLC.

We have formerly shown spontaneous cytotoxic T-cell reactivity against indoleamine 2,3 dioxygenase (IDO; refs. 6, 7), an immune regulatory enzyme that suppresses T-cell immunity. IDO catalyzes the initial and rate-limiting step in the degradation of tryptophan (Trp), which leads to local depletion of Trp and an increase in downstream metabolites, for example, kynurenine (Kyn). It has been demonstrated that patients with different tumor types have an elevated Kyn/Trp ratio compared with healthy donors, which suggests elevated IDO activity in patients with cancer (8). Moreover, the ratio of Kyn/Trp in serum has been proposed by others as a noninvasive, in vitro biomarker for evaluating the efficacy of IDO inhibitors in the clinic (9). Clinical investigation of IDO inhibition in phase I dose-escalating trials has been initiated for patients with metastatic solid tumors (10, 11).

It is well known that cancer cells as well as dendritic cells may upregulate IDO. As IDO-specific CD8+ T cells have been shown to boost immunity against tumor-associated antigens by eliminating IDO+ regulatory cells, which, in turn, has led to a decrease in regulatory T cells (Tregs; ref. 7), this justified clinical testing of the safety and efficacy of IDO-targeting vaccination. Thus, we performed a phase I vaccination study in patients with stage III/IV NSCLC testing an IDO-derived peptide vaccine applied in Montanide adjuvant.

Materials and Methods

Patients

Patients were enrolled at the Department of Oncology at Copenhagen University Hospital (Herlev, Denmark) from June 2010 to May 2012. All patients provided written informed consent before inclusion and good clinical practice (GCP) monitoring of the study was conducted. The study was performed in accordance with the Helsinki II declaration and was initiated after approval from the National Board of Health and the local Ethics Committee at the Capital Region of Denmark. The study was registered at www.clinicaltrials.gov (NCT01219348). Primary endpoint was toxicity. In addition, clinical benefit and immunity were assessed. Tumor staging was performed according to the Union for International Cancer Control classification on computed tomography (CT) scans according to Response Evaluation Criteria in Solid Tumors (RECIST) version 1.1. Clinical assessment by CT scans was performed at baseline and subsequently every third month. Antineoplastic treatments before inclusion were administered at the Department of Oncology according to standards of care. HLA typing was performed at the Laboratory of Tissue Typing at the Copenhagen University Hospital at Righospitalet (Copenhagen, Denmark). Inclusion criteria were: stage III/IV metastatic NSCLC, 18 years of age or more, stable disease (SD) after chemotherapy/tyrosine kinase inhibitor (TKI) inhibitor (any line) according to RECIST 1.1 and a World Health Organization performance status of 0–1. In total, 34 patients were screened for inclusion, only patients harboring the tissue type HLA-A2 were eligible (N = 15). The HLA-A2–negative patients (N = 10) were treated at the Department of Oncology with the standard of care for metastatic NSCLC disease. Patients defined as screening failure (failure to meet inclusion criteria) due to (prednisone treatment N = 3, progressive disease N = 4, autoimmune disease N = 1, diagnosis of prostate cancer <5 years before inclusion N = 1), were excluded from the statistical analyses. Posttrial, reevaluation of CT scans revealed that one patient treated with the vaccine did not completely meet the RECIST 1.1 definition at baseline, because this patient’s (#17) target lesion was down scaled from 1.5 to 1.3 cm. The patient is included in both the clinical and immunologic analyses. Patients were treated every second week (induction) for 2.5 months and thereafter monthly (maintenance) with imiquimod ointment (Aldara, Meda AS, www.meda.se) and vaccine (100 μg ID05 peptide, sequence ALLEIASCL, mixed with 900 μL Montanide) administered subcutaneously until progression. The imiquimod (1 sachet 5%) was applied 8 hours before vaccination and covered by a patch until vaccine administration in the same area of the skin.

Immunohistochemistry

Available formalin-fixed paraffin-embedded samples of NSCLC tumor specimens were collected for immunohistochemical (IHC) studies. IHC evaluation, on 3 μm thick sections, was performed using the IDO antibody (Anti-IDO, clone 1F8.2, Millipore) following the manufacturer’s instructions. Briefly, the sections were pretreated in PIB Link from Dako using high low pH target retrieval solution (Dako DM828). The staining was performed on the DakoLink 48 from Dako utilizing the EnVision Flex+ Detection Kit (Dako K8002). The primary antibody was diluted 1:25
using Antibody Diluent (Dako DM830) and was incubated for 20 minutes. The sections were counterstained with hematoxylin. As control for IDO staining tissue samples from placenta (syncytiotrophoblasts cells) known to stain positive for IDO were used.

**Flow cytometry**

Flow cytometry analyses were carried out on a fluorescence-activated cell sorting (FACS) Canto II cytometer (BD Biosciences). Peripheral blood mononuclear cells (PBMC) were obtained from peripheral blood by gradient centrifugation by Lymphoprep technique. Isolated cells were frozen immediately with 10% dimethyl sulfoxide and 90% humanized AB-serum and stored at −140°C.

For the CD4+/Treg assay, PBMCs were thawed and incubated with mouse serum (Invitrogen) followed by labeling with fluochrome-conjugated antibodies: PB-CD3 (DAKO), PerCP-CD4 (BD Bioscience), APC-CD25 (BD-Pharmingen), PE-CD27 (BD Pharmingen), and the relevant isotypes were used for antigen surface staining. PE-FoxP3 (ebioscience) and PE-isotype PE rat IgG2a (ebioscience) were used for intracellular staining; cells were fixed and permeabilized using a fixation/permeabilization kit (ebioscience) according to the manufacturer's instruction. As dead cell marker APC-Cy7 near surface staining for CD8+ manufacturer's instruction. As dead cell marker APC-Cy7 near surface staining for CD8+ was used. Antigen surface staining for CD8+/CD4+ Tcm, Tem, Temra, and Tnaive analyses were PB-CD3 (DAKO), PerCP-CD4 (BD Bioscience), APC-CD8 (BD-Pharmingen), PE-Cy7-CCR7 (BD Pharmingen), Krom-Orange-CD45 (Ramcon), and FITC-CD45RA (BD Bioscience). For analyses of myeloid-derived suppressor cells (MDSC) surface antigen labeling was used: FITC-HLA-DR- lineage negative (BD Pharmingen), PE-Cy7-CD3 lineage (BD Pharmingen), PE-Cy7-CD19 lineage (BD Pharmingen) and PE-Cy7-CD56 lineage (BD Pharmingen), PE-CD33 (DAKO), APC-CD11b (BD Pharmingen), PerCP-CD14 (BD Pharmingen), and PB-CD15 (Biolegend).

Tetramer staining was performed in PBS + 2% fetal calf serum (FCS) for 15 minutes at 37°C, followed by antibody staining for 30 minutes on ice. The tetramers were prepared using the MHIC-peptide exchange technology as described (12). The MHC tetramer complexes used were: HLA-A2/IDO5 ALLEIASCL -PE, HLA-A2/IDO5 ALLEIASCL-APC, HLA-A2/HIV(ILKEPVHGV) -PE and HLA-A2/HIV(ILKEPVHGV)-APC. The antibodies used for the stainings were: CD8-PerCP, CD4-FITC, CD14-FITC, CD16-FITC, CD19-FITC (BD Biosciences), and CD40-FITC (AbD SeroTec).

**Cytotoxicity assay**

Conventional 31Cr-release assays for CTL-mediated cytotoxicity were carried out as described (13). T2 cells were preloaded with 20 μmol/L peptide and 31Cr for 1 hour, then washed two times in RPMI + 10% FCS. Target cells [5,000/well] were T2 cells (American Type Culture Collection) with HIV-1 pol,347-484 (ILKEPVHGV) or T2 cells with IDO5-199-207 (ALLEIASCL). Target cells [5,000/well] were used in a cytotoxicity assay with an IDO-positive HLA-A2 cancer cell line (SW480). Cell lines included in the study were tested and authenticated by HLA genotyping. The cell lines were routinely confirmed with their HLA typing and antigen expression by flow cytometry and coculture assays, respectively.

**Elispot**

ELISPOT analyses were performed according to CIMP Immunoguiding Program (CIP) guidelines (http://cimp.eu/cimt/files/dl/cip_guidelines.pdf). PBMC were stimulated once in ex vivo medium +5% HS, 15 μmol/L peptide + 120 U/mL interleukin (IL)-2 in vitro with the IDO5 peptide before analysis to extend the sensitivity of the assay (14). Nitrocellulose bottomed 96-well plates (MultiScreen MSIPN4W; Millipore) were coated overnight with IFN-γ capture mAb (Mabtech). The wells were washed, blocked by ex vivo medium (no serum used), and the effector cells were added in duplicates at two different cell concentrations, with or without 5 μmol/L of the IDO5 peptide. The plates were incubated overnight. The following day, medium was discarded and the wells were washed before addition of the relevant biotinylated secondary Ab (Mabtech). The plates were incubated at room temperature for 2 hours, washed, and avidin-enzyme conjugate (AP-Avidin; Calbiochem/Invitrogen Life Technologies) was added to each well. Plates were incubated at room temperature for 1 hour and the enzyme substrate NBT/BCIP (Invitrogen Life Technologies) was added to each well and incubated at room temperature for 5 to 10 minutes. Upon the emergence of dark purple spots, the reaction was terminated by washing with tap water. The spots were counted using the ImmunoSpot Series 2.0 Analyzer (CTL Analyzers). Elispot responses were considered positive when the numbers of IFN-γ-secreting cells were at least 2-fold above the negative control (medium) and with a minimum of 50 spots (per 6’10e5 PBMC) detected.

**Establishment of IDO-specific T-cell cultures**

The culture was enriched for specific cells by staining PBMC with HLA-A2/IDO5-PE and HLA-A2/IDO5-APC tetramer followed by sorting of tetramer-binding cells on a FACS Aria. Specific cells were expanded by incubation with the IDO5-positive HLA-A2 (MCV) humanized AB-serum and stored at −80°C. Cells were expanded by incubation with the IDO5-positive HLA-A2 (MCV) humanized AB-serum and stored at −80°C. Cells were expanded by incubation with the IDO5-positive HLA-A2 (MCV) humanized AB-serum and stored at −80°C. Cells were expanded by incubation with the IDO5-positive HLA-A2 (MCV) humanized AB-serum and stored at −80°C. Cells were expanded by incubation with the IDO5-positive HLA-A2 (MCV) humanized AB-serum and stored at −80°C. Cells were expanded by incubation with the IDO5-positive HLA-A2 (MCV) humanized AB-serum and stored at −80°C. Cells were expanded by incubation with the IDO5-positive HLA-A2 (MCV) humanized AB-serum and stored at −80°C. Cells were expanded by incubation with the IDO5-positive HLA-A2 (MCV) humanized AB-serum and stored at −80°C. Cells were expanded by incubation with the IDO5-positive HLA-A2 (MCV) humanized AB-serum and stored at −80°C. Cells were expanded by incubation with the IDO5-positive HLA-A2 (MCV) humanized AB-serum and stored at −80°C. Cells were expanded by incubation with the IDO5-positive HLA-A2 (MCV) humanized AB-serum and stored at −80°C. Cells were expanded by incubation with the IDO5-positive HLA-A2 (MCV) humanized AB-serum and stored at −80°C. Cells were expanded by incubation with the IDO5-positive HLA-A2 (MCV) humanized AB-serum and stored at −80°C. Cells were expanded by incubation with the IDO5-positive HLA-A2 (MCV) humanized AB-serum and stored at −80°C. Cells were expanded by incubation with the IDO5-positive HLA-A2 (MCV) humanized AB-serum and stored at −80°C. Cells were expanded by incubation with the IDO5-positive HLA-A2 (MCV) humanized AB-serum and stored at −80°C. Cells were expanded by incubation with the IDO5-positive HLA-A2 (MCV) humanized AB-serum and stored at −80°C. Cells were expanded by incubation with the IDO5-positive HLA-A2 (MCV) humanized AB-serum and stored at −80°C. Cells were expanded by incubation with the IDO5-positive HLA-A2 (MCV) humanized AB-serum and stored at −80°C. Cells were expanded by incubation with the IDO5-positive HLA-A2 (MCV) humanized AB-serum and stored at −80°C. Cells were expanded by incubation with the IDO5-positive HLA-A2 (MCV) humanized AB-serum and stored at −80°C. Cells were expanded by incubation with the IDO5-positive HLA-A2 (MCV) humanized AB-serum and stored at −80°C. Cells were expanded by incubation with the IDO5-positive HLA-A2 (MCV) humanized AB-serum and stored at −80°C. Cells were expanded by incubation with the IDO5-positive HLA-A2 (MCV) humanized AB-serum and stored at −80°C. Cells were expanded by incubation with the IDO5-positive HLA-A2 (MCV) humanized AB-serum and stored at −80°C. Cells were expanded by incubation with the IDO5-positive HLA-A2 (MCV) humanized AB-serum and stored at −80°C. Cells were expanded by incubation with the IDO5-positive HLA-A2 (MCV) humanized AB-serum and stored at −80°C. Cells were expanded by incubation with the IDO5-positive HLA-A2 (MCV) humanized AB-serum and stored at −80°C. Cells were expanded by incubation with the IDO5-positive HLA-A2 (MCV) humanized AB-serum and stored at −80°C. Cells were expanded by incubation with the IDO5-positive HLA-A2 (MCV) humanized AB-serum and stored at −80°C. Cells were expanded by incubation with the IDO5-positive HLA-A2 (MCV) humanized AB-serum and stored at −80°C. Cells were expanded by incubation with the IDO5-positive HLA-A2 (MCV) humanized AB-serum and stored at −80°C. Cells were expanded by incubation with the IDO5-positive HLA-A2 (MCV) humanized AB-serum and stored at −80°C. Cells were expanded by incubation with the IDO5-positive HLA-A2 (MCV) humanized AB-serum and stored at −80°C.

**Assessment of IDO activity in patient serum**

Sera from patients were obtained at pretreatment, at the fourth and sixth vaccine and subsequently every third month. Of note, 9 mL of blood was drawn in a dry vial and spun down at 3,000 rpm in 10 minutes. Sera were aliquoted in 1.8 mL Nunc cryopreservation vials and stored at −80°C freezer.

IDO activity was estimated by quantifying Trp and its metabolite Kyn in patient sera, essentially as previously described (15, 16). Briefly, 100 μL thawed serum was diluted 1:2 with 0.05 mol/L KOP4 buffer, PH 6.0, followed by protein precipitation with TCA 2 mol/L. Trp and Kyn were then identified in 100 μL supernatant by high-performance liquid chromatography (HPLC; LC 10 AvP system, Shimadzu) using a C18 column (Reprosil-Pur Basic, Dr.

Published Online First November 11, 2013; DOI: 10.1158/1078-0432.CCR-13-1560
Maisch GmbH, Entringen, Germany) and a 3% acetonitrile (ACN) 0.05% trifluoroacetic acid (TFA) isocratic gradient over 30 minutes at a flow rate of 0.25 mL/minute. At the end of each run, the column was washed with methanol 90% and re-equilibrated with 3% ACN 0.05% TFA before injection of the next sample. Peaks were detected with a fluorescent detector (Exc. 285 nm/Em 405 nm) and a UV/Vis detector at 360 nm for Trp and Kyn, respectively (16). To quantify both products, a series of 8 standards from 5 to 100 µmol/L Trp and 1.25–20 µmol/L Kyn (both Sigma) was generated by diluting frozen stock solutions in human serum albumin 70 g/L (Biotest AG) in duplicates. Sera of three selected healthy donors were also added to each HPLC run in duplicate, showing an interassay coefficient of variation for Trp, Kyn, and Kyn/Trp ratio less than 6%. Normalization was performed by spiking 3-nitro-L-tyrosine at 0.1 mmol/L (Sigma, detection 360 nm) as internal calibrator in all samples. Results were calculated from peak areas and are expressed as Kyn µmol/L/Trp mmol/L ratios (mean of triplicate or duplicate measurements; ref. 15).

Statistical analysis
Statistical analyses were performed by GraphPad Prism software v. 5.0. Survival statistics for overall survival (OS) and progression-free survival (PFS) were performed by the Kaplan–Meier method. Survival comparisons between HLA-A2–positive and -negative patients were calculated by the log-rank test. Individual serial samples of T-lymphocyte subsets were assessed using paired Wilcoxon signed rank test. For analyses of the prelevel of Kyn/Trp subsets were assessed using paired Wilcoxon signed rank test. The log-rank test. Individual serial samples of T-lymphocyte subsets were assessed using paired Wilcoxon signed rank test. For analyses of the prelevel of Kyn/Trp subsets were assessed using paired Wilcoxon signed rank test. The log-rank test. Individual serial samples of T-lymphocyte subsets were assessed using paired Wilcoxon signed rank test. For analyses of the prelevel of Kyn/Trp subsets were assessed using paired Wilcoxon signed rank test. The log-rank test. Individual serial samples of T-lymphocyte subsets were assessed using paired Wilcoxon signed rank test. For analyses of the prelevel of Kyn/Trp subsets were assessed using paired Wilcoxon signed rank test. The log-rank test. Individual serial samples of T-lymphocyte subsets were assessed using paired Wilcoxon signed rank test. For analyses of the prelevel of Kyn/Trp subsets were assessed using paired Wilcoxon signed rank test. The log-rank test. Individual serial samples of T-lymphocyte subsets were assessed using paired Wilcoxon signed rank test. For analyses of the prelevel of Kyn/Trp subsets were assessed using paired Wilcoxon signed rank test. The log-rank test. Individual serial samples of T-lymphocyte subsets were assessed using paired Wilcoxon signed rank test.

Results
Demographics
Patients characteristics for both HLA-A2–positive patients (N = 15) and HLA-A2–negative patients (N = 10) otherwise eligible but not harboring the necessary HLA-A2 allele for vaccination are presented in Fig. 1A. According to protocol requirements, CT scans for HLA-A2–positive patients were described using RECIST 1.1, whereas for HLA-A2–negative patients, RECIST 1.0 according to local standard was used, thus baseline characteristics were not completely comparable among the two groups. Patients were in SD after chemotherapy and/or TKI treatment (any line) at the time of enrolment. Vaccine treatment was initiated at least 28 days after last dose of chemotherapy. Metastatic sites of tumor lesions as visualized by CT scan are shown in Fig. 1B; as expected the most frequent locations were in the lung and lymph nodes. Screening for brain metastasis was not routinely performed at inclusion. Four patients (26%) included in the trial eventually developed brain metastases.

Toxicity
No grade 3 or 4 common toxicity criteria adverse events (CTCAE) toxicity was observed, hence the CTCAE 1 to 2 events are shown in Fig. 1C. High frequencies of fatigue, shortness of breath, coughing, and haemoptysis were present at baseline and were probably related to the lung cancer. Nausea and headache were observed in relation to the development of brain metastases, whereas dyspepsia, abdominal pain, and diarrhea could be related to the vaccine treatment, as IDO is expressed in the epithelial cells in the gastrointestinal tract (17, 18). In 90% of the patient developments of grade 1 to 2, short-term, local reactions at the vaccine site (i.e., redness, swelling, or itching) were observed. Local treatments with steroid ointments removed the symptoms.

Clinical efficacy and survival estimates
Duration of the vaccine treatment demonstrated a mean of 7.9 months (238 days) of vaccine treatment [with a 95% confidence interval (CI) of 4.6–11.3 months] as shown in Fig. 1D. The mean number of vaccinations was 11 (range, 6–29). Clinical benefit was defined as objective response or SD for a minimum of 8.5 months after vaccine initiation, corresponding to treatment with at least nine vaccines and demonstration of SD on three consecutive CT scans. On the basis of this definition, seven of 15 patients had clinical benefit; one partial response (PR), and six SD. Tumor response to the vaccine treatment is depicted in Fig. 2A. One patient (Pt. #18) developed PR of target lesions in the liver with continuous regression from the second to the fifth evaluation (−14%, −22%, −31%, and −31%, respectively, confirmed PR) as shown in Fig. 2B. This patient is still on study (17 months+). Patients were divided into two groups: SD + PR (SD; N = 6, PR; N = 1) and PD (N = 8) for the following analyses. PFS defined as date of first vaccine treatment to date of PD is provided in Fig. 2C (top), median PFS was 5.2 month (157 days), two patients are still on study (15–17 months+). Patients in the SD+PR group (N = 7) had a trend toward longer OS (P = 0.05) than patients in the PD group (N = 8), as shown in Fig. 2C (bottom). In the vaccination group, nine patients are still alive. Among these nine patients, six belonged to the SD+PR group. OS estimate defined as date of first vaccine to date of death is shown for both vaccinated HLA-A2–positive patients (dotted line) and unvaccinated HLA-A2–negative patients (solid line) in Fig. 2D. Vaccinated patients had a median survival of 25.9 months (778 days) demonstrating significant longer OS (P = 0.03) when compared with the vaccine-untreated group of patients demonstrating a median OS of 7.7 months (237 days).

IDO expression in NSCLC
Expression of IDO in the cytoplasm of the cancer cells in pretreatment tumor biopsies was detected in nine out of ten patients. Grading of IHC staining (negative, 1+, 2+ or 3+ positive) of IDO expression was performed as suggested in a recent study on NSCLC (19). The grading of IDO expression in the tumor samples was defined as grade 1 = 0% to 30%; grade 2 > 30% to 50%, and grade 3 > 50% of the tumor cell stained, the estimates were performed by an experienced pathologist blinded to clinical response. From five patients,
we did not have access to sufficient tumor biopsies and in one patient, the IHC was inconclusive. Grading of IDO expression is shown in Fig. 3A and examples of IHC (grade 3 in Pt. #04 and Pt. #06, respectively) are shown in Fig. 3B. All accessible tumors expressed IDO, and no correlation of grading in IDO expression and clinical response to vaccine treatment was found.

Frequency of IDO-specific T cells in PBMC during vaccination

Presence of IDO-specific CD8⁺ T cells was demonstrated by IFN-γ Elispot. All assays were performed in duplicates at two different cell numbers (2.5×10⁵ and 6.0×10⁵ PBMCs/well). In Fig. 4A, Elispot responses ± IDO peptide are exemplified for patient #02 who had prolonged SD. IFN-γ Elispot IDO responses could be demonstrated in all patients at different time points as shown in Fig. 4B (left). Thus, IFN-γ-releasing cells were frequently detected both in SD+PR and PD patients during vaccinations. In Fig. 4B (right), mean IDO response for different time points are demonstrated. Only at baseline did the measurement of CD8⁺ IDO-specific T cells trend toward differing (P = 0.05) the SD+PR compared with PD patients, as shown in Fig. 4C.

Low-frequency responses of tetramer-positive IDO-specific T cells were detected after cell culturing and expansion in three SD patients postvaccine treatment, as exemplified.
for patient #03 in Fig. 4D (left). The tetramer-positive cells were isolated by cell sorting and expanded in vitro. These T cells demonstrated effective, specific killing (~20% lysis) of TAP-deficient T2 cells pulsed with the IDO5 peptide, and in addition, effective killing (~30% lysis) of an IDO+ HLA-A2–positive cancer cell line (SW480) as shown in Fig. 4D (right).

**Treg reduction after vaccine therapy**

Flow cytometry analyses of PBMCs were performed pretreatment and after fourth and sixth vaccinations in all patients. Gating strategy (shown in Supplementary Material) was based on living lymphocytes (single cells, living, CD45+ ) as being the reference population for subtypes of immune cells CD4+ and CD8+, including memory subtypes (TNaive, TCM, TEM, TEMRA), Tregs, and natural killer cells (NK) analyzed. For the monocyteic MDSCs (Mo-MDSC), monocytic cells were included in the gating on lymphocytes. Figure 5A (left) depicts the percentage CD8+ T cells during treatment. (right) the CD8+ TEM (CD3+CD8+CD45RA-CCR7+) and Fig. 5B (left) depicts the CD4+ T-cell population. In general, no significant changes were seen during treatment in these cell populations. However, the SD+PR patients experienced an increase (though insignificant) in peripheral CD8+ and in CD8+ TEM cells, which was not observed in the PD patients. Interestingly, a significant decreased level of the Tregs ($P = 0.03$) was detected after six vaccinations in both SD+PR and PD patients, depicted in Fig. 5B (right). In addition, a trend toward an increase in the NK cell population ($P = 0.05$) after six vaccinations demonstrated no difference between SD+PR and PD patients seen in Fig. 5C. The Mo-MDSC population remained unchanged during treatment as shown in Fig. 5D. Only a tendency in lower pretreatment level of MDSC was seen in SD+PR patients compared with pretreatment level in PD patients.
Stabilization of Kyn/Trp ratio suggesting IDO blockade

The elevated Kyn/Trp ratio has been suggested to mirror IDO activity hence HPLC measurements of Kyn and Trp levels from sera at baseline, fourth and sixth vaccines were performed. In Fig. 6A, the ratio of Kyn/Trp at the different time points is shown. In eight of 11 patients, the level at the sixth vaccine was stable or lower than at pretreatment with no difference between SD+PR patients (4/5) and PD patients (4/6). However, at baseline the SD+PR patients had nonsignificantly higher Kyn/Trp levels (mean 71.1; 95% CI, 35.9–106.3) compared with PD patients (mean 57.8; 95% CI, 32.9–82.8) shown in Fig. 6B. Overall no significant decrease in the Kyn/Trp ratio was measurable after the sixth vaccine (mean 68.4; 95% CI, 44.2–92.2) compared with pretreatment (mean 67.0; 95% CI, 42.8–91.2), as shown in Fig. 6C (N = 11, SD+PR; N = 5, PD; N = 6). However, only two patients [Pt. #04 (after fourth vaccine) and #06 (after sixth vaccine); see Fig. 6A and C] experienced a strong augmentation of Kyn/Trp, suggesting increased IDO activity. Interestingly, these two patients also demonstrated high IDO expressions (grade 3) in the IHC analyses and experienced early tumor progression. Measurements of the long-term Kyn/Trp ratio were performed from four patients with maintained SD as shown in Fig. 6D. Long-term stabilization was demonstrated in two clinical responders, that is, Pt. #03 (SD for 2 years) and Pt. #18 (PR ongoing). About Pt. #03, #15, and #19, they went off study due to progression within 3 months after the latest time point of Kyn/Trp measurements.

Discussion

We recently described that IDO-reactive T cells are able to recognize and kill tumor cells as well as IDO-expressing dendritic cells, that is, one of the major immune-suppressive cell populations (6, 7). Consequently, IDO may serve as an important and widely applicable target for anticancer immunotherapeutic strategies. We tested this hypothesis in a first-in-man clinical phase I vaccination trial, comprising 15 patients with advanced NSCLC were vaccinated with an HLA-A2–restricted IDO peptide formulated in Montanide. The vaccine was well tolerated with manageable side effects and no CTCAE grade 3/4 toxicities. Only local reactions at the vaccine site were frequently induced (90% of patients) most likely due to Montanide (20) and imiquimod (21).

On the basis of an expected median PFS in this patient group of approximately 6 to 7 months (22), we defined clinical benefit as objective response or SD for at least 8.5 months, which was demonstrated in 47% of the treated patients. One patient with objective response (PR) had continuous tumor regression on vaccine treatment for 1 year before qualifying as a PR. This pattern of long-term disease stabilization, which eventually can lead to objective responses, has also been shown with ipilimumab treatment, and has led to development of the immune-related response criteria (23). In our study, the median OS was more than 2 years, which was higher than expected for this patient group and is emphasized by the fact that six of seven of the SD patients are still alive. One explanation could be that T-cell priming by the IDO vaccine in some patients is of importance for response to succeeding antineoplastic treatments or induces long-lasting antitumor effects (24). To this end, the recently U. S. Food and Drug Administration-approved sipuleucel T vaccine for patients with advanced prostate cancer demonstrated prolonged OS though no effect on time to progression was demonstrated (25).

The clinical significance of HLA phenotype in patients with cancer has been widely investigated. Importantly, it was recently described in a large study that expression of HLA-A2 was an unfavorable prognostic factor in stage I NSCLC patients (26). This study underlines the potential importance of the significantly longer OS observed in vaccinated HLA-A2 patients compared with unvaccinated HLA-A2–negative NSCLC patients, though these data
need confirmation in large clinical trials. To this end, it is important to stress that the difference in OS among the two groups might have been influenced by differences in baseline characteristics in terms of tumor load, distribution of metastatic lesions, and histologic subtypes thus underscoring the need for a randomized trial. Also, it should be noted that OS is influenced by subsequent therapy; including radiation therapy, whole brain radiation for multiple brain metastases and targeted therapy such as Tarceva (erlotinib). Several cancer vaccine studies have previously indicated a possible positive influence on efficacy of subsequent therapy, for example, in a recent lung cancer study (27). Thus, it cannot be ruled out that the IDO vaccine have increased responsiveness to further therapy.

Tumor cells expressing IDO by IHC staining have been detected in many different carcinomas, including NSCLC (28). The correlation between IDO expression (IHC) and negative clinical outcome has among other tumor types been shown in ovarian cancer (29), glioblastoma (30), and endometrial cancer (31). Our data demonstrate that IDO expression of IDO was detected, which might implicate an unfavorable prognosis. Also, these two patients experienced a strong increase in the Kyn/Trp ratio.

Figure 4. Detection of IDO-specific CD8^+ T cells. A, example of IFN-γ Elispot response ± IDO peptide from Pt. #02. B, left, IFN-γ Elispot responses in the individual patients (SD = PR and PD patients marked at the graph, respectively) at different time points (mean of duplets, 6.0*10^5 PBMC/well, background spot production is subtracted). Right, mean IFN-γ Elispot response for different time points, that is, baseline (N = 15), at 4th (N = 15), at 6th (N = 15), at 9th (N = 8, mainly SD patients), and at 12th (N = 6, only SD patients; background spot production is subtracted) vaccine. C, frequency of IDO-specific CD8^+ T cells at baseline in SD versus PD patients (background spot production is subtracted). D, left, flow cytometry dot-plots of tetramer-positive IDO-specific T cells from Pt. #03 compared with a control with an irrelevant HIV peptide. Right, after T-cell culturing, cell sorting and rapid expansion effective lysis (~20%) of IDO peptide loaded T2 cells was detected as well as effective killing (~30%) of an IDO-positive HLA-A2 cancer cell line (SW480) by IDO-specific CD8^+ T cells.
T cells (magnitude and function) from PBMCs. Our data demonstrate that IDO-specific T cells were indeed detectable in all patients. This observation indicates that IDO could be a suitable target for vaccination as immunity toward IDO seems to be a general characteristic of patients with NSCLC independent of clinical status. In two SD

Figure 5. Flow cytometry analyses performed at pretreatment and after the fourth and the sixth vaccine in all patients (N = 15) white bars; SD + PR (N = 7), black bars; PD (N = 8), gray bars. Gating strategy was based on lymphocytes (single cells, living, CD45+) as the reference population for subtypes of immune cells except for the Mo-MDSC in which monocytic cells were included in the gating. A, left, changes in percentage of CD8+ T cells. Right, changes in percentage of CD8 TEM cells (%CD3+CD8+CD45RA-CCR7-). B, left, changes in percentage of CD4+ T cells. Right, changes in percentage of Treg cells (%CD4+CD25+CD127-FoxP3+). C, changes in percentage of NK cells (%CD3−CD19−CD56+CD16+). D, changes in percentage of Mo-MDSC cells (%HLA-DRlowLin-CD33+CD11b−CD14+). * = significant P value.
patients, an IDO-specific Elispot response was detected during 1 year of treatment, suggesting sustained long-term IDO reactivity. Noteworthy, we found a trend toward higher pretreatment levels of IDO-specific T cells in the SD\textsuperscript{+}PR patients compared with the PD patients. Thus, our data indicate that the SD\textsuperscript{+}PR patients had a higher level of T cells primed for an IDO response, which is in accordance with previously suggested IDO immunity (7). In addition, we utilized the MHC peptide tetramer technology to directly visualize IDO-specific T cells. In three patients, low-frequent tetramer-positive IDO-specific T cells were detectable, which was fewer compared with the number of patients with Elispot responses. Interestingly, in one patient (#03), clinical benefit (SD in 2 years) and continuously high numbers of IFN-\(\gamma\)-producing IDO-specific T (Elispot) cells were demonstrated. In addition, in the same patient, IDO-specific T cells were demonstrated by tetramer staining and these T cells exerted specific cytotoxicity toward IDO-expressing tumor cells. Overall, although IDO-specific T-cell responses were detectable, the vaccine, in general, did not seem to induce strong responses (magnitude). Although we only measured specific responses in PBMCs and not at the site of the tumor, this suggests that IDO vaccination might be more effective using other adjuvants that facilitate more powerful immune responses. Hence, the administration of a short IDO peptide formulated in Montanide is most likely not optimal, and research into the generation of enhanced CTL stimulation needs further attention. Recently, it was recognized that persisting antigen delivery at vaccine sites using Montanide induced dysfunctional T cells (32) forming the basis for development of newer rapidly degradable adjuvants (33). Other aspects include route of administration, homing of T cells to the tumor site, entry into the tumor microenvironment, and maintenance of function systemically, as well as at the tumor site.

The boosting of IDO-specific immunity could have both direct and indirect effects. First, such T cells may directly kill IDO\textsuperscript{+} cancer cells. In addition, they may function by eliminating suppressive immune and stromal cells. Hence, we performed phenotypic characterization of PBMCs to quantitate different immune cells. Noteworthy, we observed a significant reduction of the Treg population after the sixth vaccine in all treated patients. Albeit that the observed decrease in Tregs was seen in both SD\textsuperscript{+}PR and PD patients, our results show that important pharmacodynamic changes in immune cells during vaccine treatment can occur. The decrease in Tregs is in accordance with our previous observation that the "supportive" effect of IDO-specific T cells in \textit{vivo} is, in part, mediated through a decrease in the frequency of Tregs (7). The IDO pathway is linked to Treg biology because IDO-expressing dendritic cells induce the differentiation of naive CD4\textsuperscript{+} cells toward a FoxP3\textsuperscript{+} phenotype (34, 35). The observed trend of an increase in CD8\textsuperscript{+} T cells and TEM in patients with clinical benefit might also indicate reduced suppression.

Figure 6. Ratios of Kyn and Trp levels from patient sera as determined by HPLC. A, ratio of Kyn/Trp (\textmu mole/L/mole/L) from sera at baseline, at fourth and at sixth vaccine, SD\textsuperscript{+}PR (\(N = 7\)) white bars; PD (\(N = 8\)) black bars. From Pt. #02 only baseline level was obtained; from Pt. #4, #5, and #11 only sera at pretreatment and at the fourth vaccine were available; and from Pt. #03, only sera at pretreatment and after the sixth vaccine were available. B, baseline levels of the Kyn/Trp ratio in SD versus PD patients. C, ratio of Kyn/Trp at baseline compared with at sixth vaccine (\(N = 11\)). D, long-term analyses of Kyn/Trp levels in three patients with SD and one patient with PR. This graph combines experiments performed at two different time points.
The role of NK cells in regard of an antineoplastic effect in human cancer is controversial. NK cells may contribute to antitumor activity because they recognize tumor cells independent of MHC Ag expression (36). Recently, it has been suggested that IDO as part of an immune-evasion strategy does induce downregulation of cell surface NK receptor expression (37). Interestingly, we found a trend toward an increase in the NK cell population in all treated (both SD+PR and PD) patients, hence no clear correlation between clinical benefit and NK cell activity was demonstrated.

Many patients with cancer have an elevated Kyn/Trp ratio compared with healthy donors but it is still unclear whether elevation in the Kyn/Trp ratio is indicative for disease progression. Moreover, Kyn production might reflect IDO induction in different peripheral cells hence blurring the picture. Because the IDO gene is sensitive for IFN-γ response elevated Kyn/Trp ratio may also reflect unspecific inflammation (38). The presence of an "inflamed" microenvironment might be used to consider patients as candidates for immunotherapy likewise the expression of PD-L1, IDO, or presence of Tregs might necessitate agents targeting negative regulation (39). We found eight of 11 patients demonstrating stable or lower level of IDO activity after the sixth vaccine. The SD+PR patients had a trend toward a higher rate of IDO-specific T cells and higher Kyn/Trp ratio before treatment. Taken together, this points toward a higher prevaccination IDO activity in the SD+PR patients compared with the PD patients. Interestingly, in two patients with long-lasting clinical response, stable Kyn/Trp ratio was maintained at long-term pointing toward a possible role as marker of response.

Overall, about the induced Treg decrease, the possibly NK cell increase, the demonstration of IDO expression in tumor biopsies, and the Kyn/Trp ratio measurements, no clear correlation to clinical activity could be demonstrated. However, important immune changes during vaccine treatment have been demonstrated implying an immune modulating capacity of the IDO vaccine and potential fundamental immune parameters to monitor, in future, IDO-based clinical trials.

The role of IDO as a novel mechanism whereby tumors evade immune-mediated rejection and the original idea of targeting negative regulation by IDO inhibition was authored more than 10 years ago (40). Results from the first clinical trials of IDO inhibitors such as 1-methyl-D-tryptophan and INCB024360 in patients with advanced cancer settings are still awaited (10, 11). Lately, methyl-D-tryptophan and INCB024360 in patients with metastatic melanoma (NCT01219348) from the first clinical trials of IDO inhibitors such as 1-DMT and docetaxel for patients with advanced cancer settings are still awaited (10, 11). Lately, methyl-D-tryptophan and INCB024360 in patients with metastatic melanoma (NCT01219348) from the Interfaculty Institute of Biochemistry in Tubingen for providing access to the HPLC system and advice for establishing Kyn/Trp detection experiments.
References

Long-lasting Disease Stabilization in the Absence of Toxicity in Metastatic Lung Cancer Patients Vaccinated with an Epitope Derived from Indoleamine 2,3 Dioxygenase


Updated version
Access the most recent version of this article at:
doi:10.1158/1078-0432.CCR-13-1560

Supplementary Material
Access the most recent supplemental material at:
http://clincancerres.aacrjournals.org/content/suppl/2013/11/11/1078-0432.CCR-13-1560.DC1

Cited articles
This article cites 38 articles, 13 of which you can access for free at:
http://clincancerres.aacrjournals.org/content/20/1/221.full#ref-list-1

Citing articles
This article has been cited by 10 HighWire-hosted articles. Access the articles at:
http://clincancerres.aacrjournals.org/content/20/1/221.full#related-urls

E-mail alerts
Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions
To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions
To request permission to re-use all or part of this article, use this link
http://clincancerres.aacrjournals.org/content/20/1/221.
Click on "Request Permissions" which will take you to the Copyright Clearance Center's (CCC) Rightslink site.